PHAGE THERAPY: PAST, PRESENT AND FUTURE

EDITED BY: Stephen T. Abedon, Pilar García, Peter Mullany and Rustam Aminov
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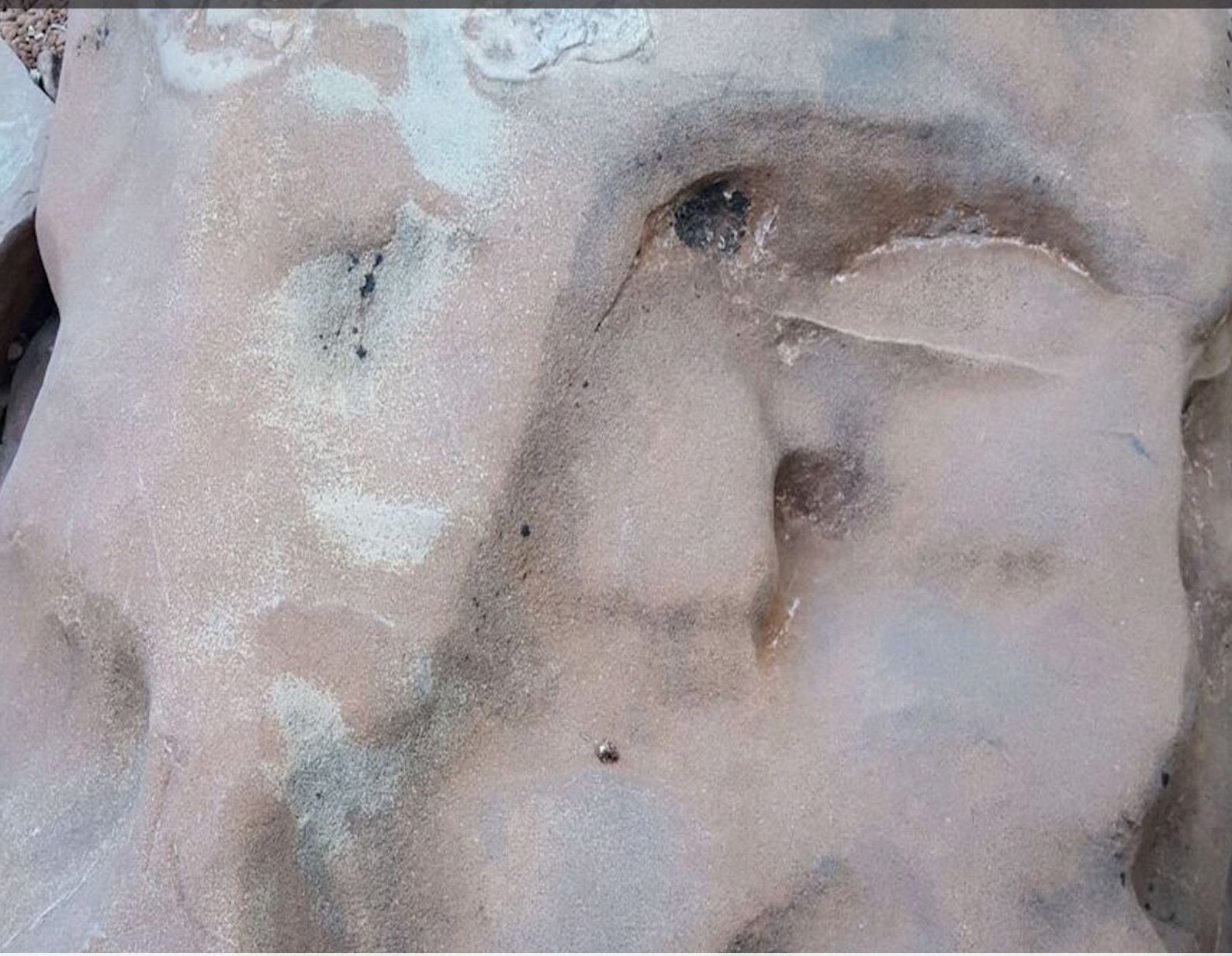

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PHAGE THERAPY: PAST, PRESENT AND FUTURE

Topic Editors:
Stephen T. Abedon, The Ohio State University, United States
Pilar García, Spanish National Research Council, Spain
Peter Mullany, University College London, United Kingdom
Rustam Aminov, University of Aberdeen, United Kingdom and Kazan Federal University, Russia

A boulder found in the bed of an arroyo (a dry creek) located in the western part of Sedona, Arizona, USA, otherwise known as West Sedona. It represents a typical, presumably somewhat microorganism denuded red rock.
Photo by Stephen Abedon

Historically, the first observation of a transmissible lytic agent that is specifically active against a bacterium (*Bacillus anthracis*) was by a Russian microbiologist Nikolay Gamaleya in 1898. At that time, however, it was too early to make a connection to another discovery made by Dmitri Ivanovsky in 1892 and Martinus Beijerinck in 1898 on a non-bacterial pathogen infecting tobacco plants. Thus the viral world was discovered in two of the three domains of life, and our current understanding is that viruses represent the most abundant biological entities on the planet.

The potential of bacteriophages for infection treatment have been recognized after the discoveries by Frederick Twort and Felix d'Hérelle in 1915 and 1917. Subsequent phage therapy developments, however, have been overshadowed by the remarkable success of antibiotics in infection control and treatment, and phage therapy research and development persisted mostly in the former Soviet Union countries, Russia and Georgia, as well as in France and Poland. The dramatic rise of antibiotic resistance and especially of multi-drug resistance among human and animal bacterial pathogens, however, challenged the position of antibiotics as a single most important pillar for infection control and treatment. Thus there is a renewed interest in phage therapy as a possible additive/alternative therapy, especially for the infections that resist routine antibiotic treatment.

The basis for the revival of phage therapy is affected by a number of issues that need to be resolved before it can enter the arena, which is traditionally reserved for antibiotics. Probably the most important is the regulatory issue: How should phage therapy be regulated? Similarly to drugs? Then the co-evolving nature of phage-bacterial host relationship will be a major hurdle for the production of consistent phage formulae. Or should we resort to the phage products such as lysins and the corresponding engineered versions in order to have accurate and consistent delivery doses? We still have very limited knowledge about the pharmacodynamics of phage therapy. More data, obtained in animal models, are necessary to evaluate the phage therapy efficiency compared, for example, to antibiotics. Another aspect is the safety of phage therapy. How do phages interact with the immune system and to what costs, or benefits? What are the risks, in the course of phage therapy, of transduction of undesirable properties such as virulence or antibiotic resistance genes? How frequent is the development of bacterial host resistance during phage therapy? Understanding these and many other aspects of phage therapy, basic and applied, is the main subject of this Topic.

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Editorial: Phage Therapy: Past, Present and Future

Stephen T. Abedon[1], *Pilar García*[2], *Peter Mullany*[3] *and Rustam Aminov*[4]*

[1] Department of Microbiology, The Ohio State University, Mansfield, OH, United States, [2] Spanish National Research Council, Villaviciosa, Spain, [3] Department of Microbial Diseases, Eastman Dental Institute, University College London, London, United Kingdom, [4] School of Medicine and Dentistry, University of Aberdeen, Aberdeen, United Kingdom

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Editorial on the Research Topic

Phage Therapy: Past, Present and Future

INTRODUCTION

As an ancient proverb states, "The enemy of my enemy is my friend." The so-called strictly lytic or virulent bacteriophages (phages)—especially the viruses of pathogenic bacteria—can certainly be considered enemies of "bad" bacteria and thereby our friends. The phage potential as antibacterial agents was recognized almost immediately upon the first generally accepted descriptions of these viruses as transmissible bacteriolytic entities (Abedon et al., 2011). As this was prior to Fleming's (1929) discovery of naturally occurring antibiotics, rather than being named as variations on that theme, the Greek concept of "phage" was chosen instead (d'Hérelle, 1917). "Phage" seemingly is a description of the macroscopic impact these viruses have on bacteria, which to the eye appear to be "eaters" or "devourers" of bacterial *cultures* (Summers, 1991), in broth or solid media.

The therapeutic, antibacterial application of phages came to be known as phage therapy, especially in clinical or veterinary contexts. More broadly, phages have also been used as biological control agents, reducing bacterial loads in foods, e.g., such as of *Listeria monocytogenes* in food processing (Bai et al., 2016), of zoonotic pathogens in food animals (Atterbury, 2009), or, in the treatment of crops against plant pathogenic bacteria as reviewed by Buttimer et al. Furthermore, modified phages can be used as DNA, protein, or drug delivery vehicles (Clark et al., 2012), and non-bacterial viruses can be used as biological control agents as well (e.g., Hyman et al., 2013; Kondo et al., 2013; Gilbert et al., 2015). Phage study, whether ultimately for therapy or biocontrol, spans from purely clinical observation to molecular analysis to considerations of immunology as well as ecology, the latter as phages represent essentially "living" drugs. In addition is the development of enzybiotics, which are therapeutic enzymes and most prominently include phage endolysins. The latter are proteins which phages employ to lyse the bacteria they are infecting, thereby releasing intracellularly produced phage progeny (Fischetti, 2008).

This diversity of studies and approaches to antibacterial therapy is important since, despite ~100 years of phage and phage therapy study (Abedon et al., 2011), there is still much to learn about phages and their use as therapeutic agents. There is also a compelling need for new safe and effective selectively toxic antibacterials, especially in the face of the antibiotic resistance crisis (Aminov, 2010). Phages and their products thus represent a largely untapped supply of such antimicrobials. Their use, however, has not yet been broadly embraced by the modern medical establishment. Exceptions are found especially in the countries of Georgia, Poland, and Russia, where phage therapy has been practiced by clinicians for many decades (Kutter et al., 2015; Cooper et al.).

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Edited by:
Joshua D. Nosanchuk, Albert Einstein College of Medicine, United States

Reviewed by:
Joshua D. Nosanchuk, Albert Einstein College of Medicine, United States
David Van Duin, Cleveland Clinic Lerner College of Medicine, United States

***Correspondence:**
Rustam Aminov
rustam.aminov@gmail.com

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In this topic, we present 37 articles on or related to the use of lytic phages as antibacterial agents. These are grouped into several distinct categories, including (i) phage isolation for phage therapy, (ii) host range characterization, (iii) other *in vitro* phage characterizations, (iv) *in vivo* phage characterization, (v) characterization of phage *therapy* in animals, (vi) phage impact on bacterial biofilms, (vii) enzybiotics, (viii) clinical phage therapy, (ix) biological control of bacteria using phages, and (x) the current state of phage therapy implementation (**Figure 1**).

PHAGE ISOLATION FOR PHAGE THERAPY

Key to any successful drug development is its discovery and subsequent characterization. For phage therapy, equivalent steps should be taken, including determination of how to combine phages into multi-phage mixtures known as phage cocktails. The review article in this topic by Weber-Dąbrowska et al. discusses the essential steps involved including sources and methods of phage isolation, choice of phage-propagation hosts, methods of characterization, selection criteria for therapeutic purposes, and limitations on phage procurement for therapy.

The use of phages as antibacterial therapeutics is especially important for targeting those pathogens for which antibiotic treatment options are limited. On-demand isolation of corresponding phages can be achieved via the enrichment of samples from environmental reservoirs, as explored by Mattila et al. Interestingly, the efficiency of enrichment-based phage isolation from municipal sewage varies considerably, with the best results seen for *Pseudomonas aeruginosa*, *Salmonella*, and the extended spectrum β-lactamase (ESBL) producing

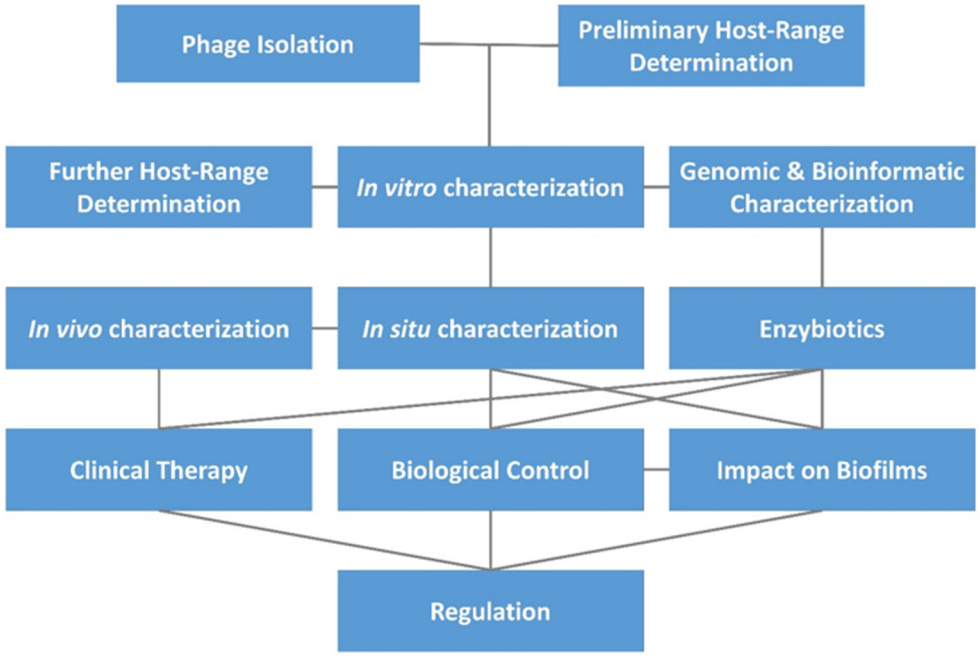

FIGURE 1 | Topics addressed in this editorial. Connections are indicated via horizontal, vertical, and diagonal lines, and initial steps are found at the top of the figure. Consideration of time and resources required by each step is beyond the scope of this editorial, though individual aspects are considered in articles as cited in the main text. In summary, phage isolation is typically done in combination with preliminary host-range characterization, i.e., as in terms of enrichment and isolation hosts. This is followed by *in vitro* characterization in association with further host-range characterization (i.e., involving a larger panel of potential hosts) and bioinformatic (*in silico*) characterization. Enzybiotic development, if undertaken, typically will follow host-range and *in silico* characterization. For promising phages, *in situ* characterization comes next, including animal models for potential human treatments (*in vivo* characterization), or with other species for non-human treatments. Clinical testing can follow, including treatment of non-human species. Alternatively, phages may be employed for biological control of environments, and both biological control and therapeutic use of phages can be against biofilms. Not only may whole phages be used for therapy or control but so too may enzybiotics. Further development toward successful commercial or public-sector implementation generally must address regulatory requirements.

Escherichia coli and *Klebsiella pneumoniae*. The procedure is less efficient for vancomycin-resistant *Enterococcus* and *Acinetobacter baumannii*, while isolation of new phages against methicillin-resistant *Staphylococcus aureus* (MRSA) strains was very difficult. Potentially, the latter may be due to the choice of environmental reservoir used for the anti-MRSA phage isolation since, as Wang et al. show, pig fecal sewage may be a better source for these phages.

HOST RANGE CHARACTERIZATION

Prior to animal testing there are various approaches toward characterizing phages for antibacterial effectiveness (Weber-Dąbrowska et al.). Most important is the range of bacteria targeted (Mirzaei and Nilsson, 2015). As a minimal requirement for phage therapy, a phage should be able to infect the bacterial isolates it is supposed to be targeting, and to display reasonable specificity so that non-target bacteria are not affected. A proper understanding of phage host range is also necessary for the development of efficient cocktails, which ideally would be formed using multiple phages that possess synergistic properties, particularly in terms of host range, thereby offering better infection control capability. Nevertheless, for some phage applications such as phage therapy and phage-based biosensors, it should be taken into account that host range is not a fixed property, but rather it can evolve over time, thereby changing phage specificity (Ross et al., 2016).

For obvious reasons, multidrug-resistant (MDR) pathogens are a primary target for phage therapy. The host range of four phage cocktails that are approved and commercially available in Georgia have been tested by Gundogdu et al. on a panel of 142 clinical strains of *E. coli* isolated in Turkey and possessing extended-spectrum β-lactamase activity. The phage cocktail antibacterial efficiencies varied from 59.2 to 87.3% of strains, as based on spot testing, which is promising given that these were difficult-to-treat MDR bacterial strains.

In addition, and like antibiotic therapy, phage therapy can result in the evolution of bacterial resistance. Understanding resistance development is important in terms of both basic biology and phage-based applications. Some phage resistant bacteria are less fit than their phage-sensitive parents. Lim et al. found that phage PB1-resistant *P. aeruginosa* displayed small-colony variants which were impaired in biofilm formation, were more antibiotic sensitive, displayed decreased twitching motility, and had reduced elastase and pyocyanin production.

OTHER *IN VITRO* PHAGE CHARACTERIZATIONS

In addition to the assessment of host range (previous section), other phage "organismal" characteristics such as burst size, ability to display lysogeny, and general plaque morphology should be evaluated. *In vitro* characteristics also include the ability to degrade experimental bacterial biofilms (subsequent section) along with complete genome sequencing. The latter typically is followed by *in silico* analyses, especially to exclude phages carrying bacterial virulence factor genes. Also, it is advantageous to exclude phages carrying lysogeny-associated genes. Hamdi et al. isolated five phages that infect *Citrobacter freundii* which they found had no known virulence factor or integrase genes. The latter are employed by many phages to initiate lysogenic cycles. Such properties suggest potential utility for these phages as antimicrobial agents.

Bardina et al. isolated and characterized three phages (UAB_Phi20, UAB_Phi78, and UAB_Phi87) infecting *Salmonella* to reduce the presence of this zoonotic bacterium in poultry. Sequence analysis of genomes did not indicate the presence of virulence factor or antibiotic resistance genes. Phage UAB_Phi20, however, encodes lysogeny-associated genes, although no lysogens could be isolated. The authors suggest that this could be because of a lack of signals needed to transcribe the CI repressor gene required for establishment of lysogeny.

Lytic phage development also depends on the physiological state of the host. According to Bryan et al. T4 phages infecting stationary phase *E. coli* may enter a "hibernation" mode, which is a persistent but reversible dormant state. Infected bacteria continue to produce some phage proteins, but phage development is halted until appropriate nutrients become available. A "scavenger" mode is encountered when exposed to limited nutrients, with the production of small quantities of progeny per infection. These considerations are important in understanding phage therapy of bacteria displaying varied physiological states, such as within biofilms or during chronic bacterial infections.

IN VIVO PHAGE CHARACTERIZATION

By *in vivo* we mean *in situ* phage assessment within other organisms or surrogates, such as during animal testing (further considered in a subsequent section). Such assessment includes in terms of safety to the host during treatment, though in practice few side effects with phage therapy have been detected (Miedzybrodzki et al.). Potential cytotoxic effects can also be evaluated using eukaryotic cell lines via different assays such as trypan blue, staining with Hoechst and propidium iodide, lactate dehydrogenase release, and the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, as described in this topic by Henein et al.

Important as well are phage and especially virion interactions with immune systems, which is a concern for biologics generally, i.e., protein-based drugs. In the article by Mirzaei et al. several *E. coli* phage preparations, were found to induce strong cytokine-driven inflammatory responses in HT-29 and Caco-2 intestinal epithelial cells. Whether this was the effect of phages *per se* or residual contaminants in phage preparation(s) was questioned by Dufour et al., however. As a Response, Mirzaei et al. proposed morphological differences as possible bases of contradictory outcomes, perhaps highlighting a need for better standardization of approaches. Mirzaei et al., in a subsequent Corrigendum, acknowledged that at least some aspect of the cytokine responses described in the original publication may have been due to residual contaminants.

For lipopolysaccharide (LPS)-activated monocytes, neither purified phage T4 nor T4 lysate, according to the results of Bocian et al., had a significant impact on the *ex vivo* human immune response. Phage lysates however, may affect the differentiation of human monocytes into myeloid dendritic cells, but purified phage preparations do not have that effect (Bocian et al.). Also regarding LPS, Miernikiewicz et al. found that recombinant short tail fiber (gp12) from phage T4 decreased inflammatory responses to LPS in a murine model. Cell culture and mouse testing indicated no toxicity, suggesting that this recombinant protein potentially could be used as an anti-LPS medicinal.

No significant increase in antiphage antibodies in the sera of most patients undergoing anti-staphylococcal phage therapy were detected by Żaczek et al. In patients with the increased titers of antiphage IgG and IgM to these phages, no interference with phage therapy clinical outcomes were observed. While the influence of purified T4 and A3/R phages on differentiation of human myeloid dendritic cells (DCs) from monocytes is negligible, phage-lysed bacterial material has a substantial effect on their differentiation (Bocian et al.). Thus, the products of phage-induced lysis of bacteria during phage therapy could influence the differentiation as well as potentially the functions of DCs that are differentiating from monocytes recruited to sites of infection.

CHARACTERIZATION OF PHAGE THERAPY IN ANIMALS

In the modern era, clinical use of drugs typically is preceded by animal testing. Phage therapy, since it has been in practice for so long, comes from a tradition where clinical use has tended to take precedence over animal testing (Abedon, 2015c). Phage therapy in the modern era nonetheless has to adopt current standards of drug development, that is, in which animal testing by necessity precedes clinical use, and several articles in the topic are devoted to animal testing of phages and phage preparations.

Wang et al. characterized the staphylococcal phage SLPW. Treatment of intra-abdominal MRSA infections in mice with phage SLPW provided high protection (80% survival) as well as reduction of infection-induced inflammatory cytokines, thus substantiating this phage as a potential therapeutic agent against MRSA infections. With a *Clostridium difficile* target, a 4-phage cocktail was tested in a *Galleria mellonella* larva model and was found by Nale et al. to be as effective as vancomycin. Another problematic multi-drug resistant nosocomial pathogen, *A. baumannii*, was targeted using phage vB-GEC_Ab-M-G7 by Kusradze et al. In a rat wound model, this phage substantially decreased bacterial loads.

Abedon briefly reviewed in a general commentary a rabbit staphylococcus osteomyelitis model system published by Kishor et al. (2016). Presented as well is a summary of several animal presumptive chronic infection models previously used for phage therapy development. A series of criteria are suggested for confirmation that such systems represent adequate disease models including demonstration of antibiotic tolerance by infecting bacteria and/or of presence of biofilms.

Pharmacological issues of phage therapy include phage transit from the stomach to the distal gastrointestinal tract. Międzybrodzki et al. showed in a rat model that modification of the stomach environment using the drugs ranitidine and omeprazole, which reduce production of stomach acid, protect staphylococcal phage A5/80, allowing passage to the lower intestine. These authors also found that phage penetration from oral administration to systemic circulation can differ among phage types as phage A5/80 reaches the bloodstream following oral administration aided by acid-reducing drugs but similarly administered T4 did not.

PHAGE IMPACT ON BACTERIAL BIOFILMS

Formation of biofilms during bacterial infection is one of the major problems in infection control. Bacteria in biofilms are extremely resistant to antimicrobials, well protected from host defenses, and tend to develop chronic infections (Cooper et al., 2014). Some bacteriophages penetrate biofilms and this may supplement or replace a less efficient antibiotic treatment (Abedon, 2015a,b). *C. difficile*, for example, produces biofilms which contribute to its virulence and impair antimicrobial activity. Nale et al. found that a cocktail of *C. difficile* phages could significantly reduce these biofilms and prevent colonization when used either alone or in combination with vancomycin.

Catheter-associated urinary tract infections (CAUTIs) such as caused by *Proteus mirabilis* are very difficult to treat as they form biofilms that are highly tolerant to antibacterials. Two novel virulent phages active against *P. mirabilis* were isolated, characterized, and studied for application on catheter-associated biofilms by Melo et al. In a dynamic biofilm model simulating CAUTIs, the authors demonstrated a significantly lower rate of *P. mirabilis* biofilm formation up to 168 h following catheterization, thus highlighting the potential of these phages in preventing bacterial surface colonization.

Biofilms can also be targeted by degrading the matrix in which bacterial cells are suspended. Gutiérrez et al. tested a recombinant protein from a staphylococcal phage encoding an exopolysaccharide depolymerase, a kind of enzybiotic. In polysaccharide producing staphylococci the enzyme can prevent and disperse biofilms, thus potentially allowing better antimicrobial access to targeted bacteria.

ENZYBIOTICS

Purified antibacterial enzymes have been described as enzybiotics (Veiga-Crespo et al., 2007), i.e., as derived from 'antibiotic'. These can include extracellular polymeric substance (EPS) depolymerases (as above) but also, phage-encoded lytic enzymes, i.e., lysins. Though some lysins are virion-particle associated, as are many EPS depolymerases (Pires et al., 2016), the majority are endolysins, meaning "from-within cell-wall degrading enzymes." Enzybiotics upon purification, however, are applied from without.

The peptidoglycan of Gram-positive bacteria is not protected by an outer membrane so is directly susceptible to phage lysins

applied from without. Blazquez et al. generated a novel ("tailor-made") endolysin (PL3) targeting *Streptococcus pneumoniae*. It combines the amidase activity of a phage endolysin (Pal) with that of LytA, a *Streptococcus* autolysin. Joining these two unrelated catalytic domains into a single protein resulted in greater antibacterial activity in a zebrafish model.

In Gram-negative bacteria, phage lysins typically need to be modified to penetrate the outer membrane barrier. This can be done by engineering hybrid molecules that combine natural lysin with an antimicrobial peptide. Yang et al. found that one such construct, PlyA, displayed good activity against growing cultures of both *A. baumannii* and *P. aeruginosa*, but not against stationary phase cells unless used with outer membrane permeabilizing agents. No antibacterial activity, however, could be detected in some bio-matrices such as culture media, milk, or sera, suggesting a need for further optimization. Endolysins such as ABgp46, as characterized by Oliveira et al., are also active against *A. baumannii,* including MDR strains. In addition, the range of activity of this lysin can be extended to other Gram-negative bacteria if used in combination with outer membrane permeabilizing agents. Endolysin LysABP-01 from *A. baumannii* phage ØABP-01 also possesses antibacterial activity against *A. baumannii* and *P. aeruginosa* as well as *E. coli*, which as shown by Thummeepak et al. can be enhanced in the presence of the antibiotic colistin.

CLINICAL PHAGE THERAPY

Clinical phage therapy is the treatment or prevention of infections in humans and the use of phages in microbiome modification. In addition is the related use of phages to treat or prevent infections in animals. Clinical phage therapy is permitted for routine use in a limited number of countries though the corresponding data from these efforts is limited. Because of the long-term treatment requirements of chronic conditions such as cystic fibrosis, the appearance of bacterial resistance to phages can be a problem. Krylov et al. propose to employ a combinatorial approach during treatment of drug-resistant *P. aeruginosa* to circumvent this problem, by using phages with a proven safety record combined into cocktails.

Although not life-threatening, some chronic skin infections, such as caused by *Propionibacterium acnes*, can require long-term antibiotic treatment, thus contributing to dysbiotic changes in microbiomes and selection for antimicrobial resistance. Phage therapy of acne may be a valuable alternative to reduce the overuse of antibiotics in the treatment of this condition, as reviewed by Jonczyk-Matysiak et al.

BIOLOGICAL CONTROL OF BACTERIA USING PHAGES

In the review article by Buttimer et al., phage biocontrol of bacterial crop diseases is compared to chemical control measures. Phages they suggest are more environmentally friendly, can be tailored against specific disease-causing bacteria, and can be easily reformulated if resistance develops. Some field trials, for example, have shown potential for phage biocontrol of bacterial blight of leek, as explored by Rombouts et al.

Another aspect of biocontrol (vs. phage therapy in the strictest sense) is reduction of loads in animals of what otherwise could be food-borne pathogens. In poultry, Ahmadi et al. found that the prophylactic administration of phage PSE, active against *Salmonella enterica* serovar Enteritidis, significantly reduced shedding of this pathogen. Improved biocontrol measures against *Salmonella* will include the selection of phages that can infect a broader range of bacterial strains. This has been explored, including in terms of phage genomics, by Bardina et al.

In another example of biocontrol, Hernández reported that bacteriophages against *Serratia* spp., which can spoil Atlantic horse mackerel (*Trachurus trachurus*), were isolated and tested for protection of fresh filets. Reductions in *Serratia* counts of more than 90% were observed in treatment with about 10^8 phages per gram of filet after 6 days of refrigerated storage (6°C). Phage application at lower densities was less effective.

THE CURRENT STATE OF PHAGE THERAPY IMPLEMENTATION

An important aspect of drug utility is availability, and this requires adequate manufacturing, marketing, and delivery to as well as education of users. Prior to these steps, it is necessary to maintain an adequately robust development pipeline along with strategies toward regulatory approval. Presently, phage therapy is relatively extensively used only in three countries, Georgia, Poland, and Russia, while its acceptance and re-implementation in other countries is still pending (Expert Round Table on Acceptance and Re-Implementation of Bacteriophage Therapy, 2016). As discussed by Cooper et al., difficulties with acceptance are due to: (i) differences in biological, physical, and pharmacological properties of phages compared to conventional antimicrobials, (ii) the need to employ multiple phage isolates (cocktails) due to the high specificity of phages (thereby allowing for more effective presumptive treatment, that is, treatment which is initiated prior to precise diagnosis of microbial etiologies), and (iii) current approval processes for antimicrobial agents that are based on chemically derived drugs and which consequently are less suitable for phages. Alternative approval pathways may be required for phage therapy (Aminov et al., 2017), while phage-derived enzybiotics are already suitable for the current approval processes as therapeutic proteins.

Based on the wealth of data obtained by some phage therapy centers, Górski et al. suggest that it is time to consider phage therapy benefits in their entirety, including compassionate use targeting cohorts of patients for whom no alternative treatment is currently available. The effects of phage therapy, which target infectious agents and which also can modulate the immune system, resemble the effect of antibiotics, which, in addition to antibacterial activity, can display other regulatory effects on the human body (Aminov, 2013). Thus, the impact of phages beyond intended antibacterial activity should be carefully evaluated in association with more standard practices of phage therapy

development. Lastly, Nagel et al. point out that as infectious diseases significantly affect developing countries, phage therapy considering its relative technical simplicity as well as ease of phage isolation, characterization, and production, could be especially useful in these settings.

CONCLUSION

Bacteriophages have been instrumental in the development of modern biology, particularly the understanding of biological process at the molecular level which has been crucial for the development of modern biological sciences (Cairns et al., 1966; Summers, 1999). They have also been used therapeutically for ∼100 years, with a good safety record (although their exploitation in this regard has lagged behind their use in molecular biology). Publications demonstrating the safety of phage applications (some of which include phase I safety trials) include (Rhoads et al., 2009; Wright et al., 2009; Miedzybrodzki et al., 2012; Sarker et al., 2012, 2016; Rose et al., 2014; Fish et al., 2016; Speck and Smithyman, 2016). Nonetheless, and despite a demonstrated need for new, safe antibacterial agents (Aminov, 2016), phage use by most Western physicians has not yet caught on, and this is due (presumably) to a lack of familiarity with phage therapy, but also because of a relative lack of regulatory approval. This volume provides an overview of a substantial number of facets of use of phages and their products in a medical and especially antibacterial context. We believe, in the face of the looming antibiotic crisis, that this approach deserves serious consideration. Hopefully phages can prove as revolutionary in the medical field as they have in the scientific.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Bacteriophages and Bacterial Plant Diseases

*Colin Buttimer[1], Olivia McAuliffe[2], R. P. Ross[3], Colin Hill[3], Jim O'Mahony[1] and Aidan Coffey[1]**

[1] Department of Biological Sciences, Cork Institute of Technology, Cork, Ireland, [2] Moorepark Food Research Centre, Teagasc, Fermoy, Ireland, [3] Alimentary Pharmabiotic Centre, University College, Cork, Ireland

Losses in crop yields due to disease need to be reduced in order to meet increasing global food demands associated with growth in the human population. There is a well-recognized need to develop new environmentally friendly control strategies to combat bacterial crop disease. Current control measures involving the use of traditional chemicals or antibiotics are losing their efficacy due to the natural development of bacterial resistance to these agents. In addition, there is an increasing awareness that their use is environmentally unfriendly. Bacteriophages, the viruses of bacteria, have received increased research interest in recent years as a realistic environmentally friendly means of controlling bacterial diseases. Their use presents a viable control measure for a number of destructive bacterial crop diseases, with some phage-based products already becoming available on the market. Phage biocontrol possesses advantages over chemical controls in that tailor-made phage cocktails can be adapted to target specific disease-causing bacteria. Unlike chemical control measures, phage mixtures can be easily adapted for bacterial resistance which may develop over time. In this review, we will examine the progress and challenges for phage-based disease biocontrol in food crops.

Keywords: bacteriophages, plant diseases, biocontrol, biopesticides, phytopathogens

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****Correspondence:***
Aidan Coffey
aidan.coffey@cit.ie

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IMPORTANCE OF CROP DISEASES

The human population is expected to reach 9.6 billion by 2050 and this will result in increased demands for food. It has been estimated that the global food supply may need to grow by as much as 70% in order to meet these demands (UN, 2013). For such growth, it has been predicted that crop supply may have to increase as much as 80–110% (Ray et al., 2013). To achieve these yields, the impact of crop disease has to be reduced. It has been estimated that at least 10% of global food production is lost to plant diseases (Strange and Scott, 2005). The major pathogens of plants are parasitic plants, oomycetes, nematodes, viruses, fungi and bacteria. Among the latter, there are over 200 plant pathogenic bacterial species (Considine and Considine, 1995). Those considered to be the

most important belonging to the genera of *Pseudomonas, Ralstonia, Agrobacterium, Xanthomonas*, *Erwinia*, *Xylella*, *Pectobacterium*, and *Dickeya* (Mansfield et al., 2012).

BACTERIOPHAGES, THEIR LIFE CYCLES AND THEIR MORPHOLOGY

Bacteriophages (phages) are the most abundant biological entity in the biosphere with an estimated number of 10^{31}, as total prokaryotic cell numbers are understood to be around 10^{30} in the biosphere and phage numbers are believed to be at least 10 times greater than this value (Whitman et al., 1998; Wommack and Colwell, 2000). Phages are specific viruses of bacteria that subvert the metabolism of their bacterial hosts in order to replicate. Of the phages that have been identified, the majority belong to the tailed phages; and these form the Taxonomic Order: *Caudovirales* (Ackermann, 2007). These phages possess icosahedral heads containing genomes comprised of double stranded DNA. The order *Caudovirales* is made up of three phage families; *Myoviridae* which have rigid contractile tails, *Podoviridae* with short, non-contractile tails and *Siphoviridae* with long flexible tails. Phages belonging to other families have highly variable morphologies with genomes of varying nucleic acid composition.

HISTORY OF BACTERIOPHAGES AND THEIR USE AS ANTIBACTERIAL AGENTS TOWARD PLANT DISEASES

The discovery of bacteriophages is credited to Frederick Twort (Twort, 1915) and Felix d'Herelle (d'Herelle, 1917). Similar findings of antibacterial agents that hinted on the existence of phage had been made prior to that of Twort and d'Herelle (Abedon et al., 2011). However, they were the first to suggest this phenomenon as being viral in origin. The potential of phages as antibacterial agents was quickly recognized, with d'Herelle in 1919 demonstrating the capability of his phage preparations to treat dysentery patients in the Hôpital des Enfants-Malades in Paris (Wilkinson, 2001). Following this work, many early studies and attempts were made to use phages to treat staphylococcal infections, cholera and bubonic plaque of humans (Sulakvelidze et al., 2001). This pre-antibiotic era approach became known as bacteriophage therapy. Studies were also initiated with the aim of using phages to control plant diseases. Mallmann and Hemstreet (1924) showed that the filtrate of decomposing cabbage could be used to inhibit the "cabbage-rot organism" *Xanthomonas campestris pv. campestris*. In 1925, Kotila and Coons demonstrated with bioassays that they could use phage to prevent soft rot by *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* ssp *carotovorum* on slices of potato tuber and carrot, respectively (Coons and Kotila, 1925; Kotila and Coons, 1925). The first field trials were also done by Thomas (1935), who showed that he could reduce the incidence of Stewart's wilt disease by treating seeds with phage against the phytopathogen *Pantoea stewartii* from 18% (untreated) to 1.5% (treated). However, this type of research became neglected as understanding of the nature of phage was poor at the time, and data on their efficacy was limited (Okabe and Goto, 1963).

BACTERIOPHAGE TYPES USED FOR THERAPY/BIOCONTROL

From a terminology perspective, the term bacteriophage therapy is usually reserved for human and animal applications. For plants the term bacteriophage biocontrol is more often used. In recent years, several studies have been published on phage biocontrol on a number of important bacterial plant pathogens, with many very promising results (see **Table 1**). The main deciding factor whether a phage is applicable for biocontrol (and also therapy in humans or animals) is whether a phage is exclusively lytic (virulent) or instead temperate in nature. Virulent phages are those which conduct infections that ultimately result in lysis of their host bacterium with the release of progeny phage particles. Temperate phages can follow the lytic route of infection but also follow the route of lysogeny, where the phage genome integrates into the bacterial chromosome or persists as a plasmid. In this form the phage is known as a prophage (Łobocka et al., 2004). With this strategy, the phage genome replicates as part of the bacterial genome of its host until a trigger switches it into the lytic cycle. These triggers can be chemical or physical (UV light or heat) in nature (Brunner and Pootjes, 1969; Müller et al., 2012). It is interesting to note that certain plant extracts can also trigger these events (Sato, 1983). Often, prophage DNA can increase the fitness of the bacterial host due to genes present on prophage genome. For example, in the case of plant pathogens, the *P. atrosepticum* prophages ECA41 and ECA29 both improve the motility of the bacterial host (Evans et al., 2010). Prophages may also harbor genes for toxins, e.g., shiga, cholera, and diphtheria toxins (Abedon and Lejeune, 2005). Another concern with these phages is the spread of virulence genes by transduction, where these phages can excise themselves from their host genomes incorporating host DNA into their own genomes facilitating horizontal transfer of genetic material among bacteria (Griffiths et al., 2000). Also, some lytic bacteriophages are capable of transduction, where they accidently pack bacterial DNA into their own capsid heads during the later stages of lytic cycle (Klumpp et al., 2008). There is also a third mechanism of phage-host interaction identified in filamentous phages (*Inovirus* family). Here, phages form a non-lethal chronic infection with continuous production of progeny phages. However, suitability of these phages for biocontrol is questionable as their infection can have varying effects on host virulence, as shown with phytopathogen *Ralstonia solanacearum* with its phage ϕRSS1 causing increased virulence (Yamada, 2013), although it has been shown possible to isolate virulent filamentous phage (Kuo et al., 1994). Another undesirable property in a phage intended for biocontrol is the ability to bring about superinfection exclusion to its host during infection. This prevents secondary infection of the host by another phage (Lu and Henning, 1994).

TABLE 1 | Summary of bacteriophage biocontrol experiments which have been conducted since the year 2000 to the present.

Pathogen	Host	Disease	Information	Reference
Pectobacterium carotovorum ssp. carotovorum, *Pectobacterium wasabiae,* *Dickeya solani*	Potato	Soft rot	Bioassays with phage ΦPD10.3 and ΦPD23.1 could reduce severity of soft rot of tubers by 80% on potato slices and 95% with whole tubers from a mixed pathogen infection.	Czajkowski et al., 2015
Dickeya solani	Potato	Soft rot/Blackleg	Phage vB_DsoM_LIMEstone1 and vB_DsoM_LIMEstone2 reduced soft rot of inoculated tubers in bioassays and in field trials which produced a potato crop with higher yields.	Adriaenssens et al., 2012
Dickeya solani	Potato	Soft rot	Bioassays with phage ΦD1, ΦD2, ΦD3, ΦD4, ΦD5, ΦD7, ΦD9, ΦD10, ΦD11 could reduce incidence of soft rot by up to 30–70% on co-inoculated potato slices with pathogen and phage.	Czajkowski et al., 2014
Streptomyces scabies	Potato	Common scab	Seed tubers treated with phage Φ*A*S1 resulted in producing tuber progeny with reduced levels of surface lesion of scab (1.2%) compared with tubers harvested from non -treated seed tubers (23%).	McKenna et al., 2001
Ralstonia solanacearum	Tomato	Bacterial wilt	Tomato plants treated with phage ΦRSL1 showed no symptoms of bacterial wilt during the experimental period; whereas all untreated plants showed wilting 18 days post infection.	Fujiwara et al., 2011
Ralstonia solanacearum	Tomato	Bacteria wilt	Simultaneous treatment of phage PE204 with *R. solanacearum* of the rhizosphere of tomato completely inhibited bacterial wilt. However, pre-treatment with phage before the inoculation of pathogen was not effective with control of bacterial wilt, whereas post treatment of PE204 delayed disease development.	Bae et al., 2012
Xanthomonas campestris pv. vesicatoria	Tomato	Bacterial spot	Greenhouse experiments with formulated phage cocktails could reduce disease severity with formulated phage cocktails providing better protection in comparison to unformulated. A similar effect was found in three consecutive field trials.	Balogh et al., 2003
Xanthomonas campestris pv. vesicatoria	Tomato	Bacterial spot	In field experiments phage treatment was comparable to disease control with copper-mancozeb. Combination of phage and plant activator (ASM) resulted in enhanced control.	Obradovic et al., 2004
Xylella fastidiosa	Grapevines	Pierce's Disease	*X. fastidiosa* levels in grapevines were significantly reduced on pre and post inoculation of a four phage (*Sano, Salvo, Prado and Paz)* cocktail. Pierce disease symptoms could be stopped using phage treatment post infection as well as applying phage prophylactically to grapevines.	Das et al., 2015
Xanthomonas axonopodis pv. *allii*	Onion	*Xanthomonas* leaf blight of onion	Field trial showed that weekly and biweekly applications of phage could reduce disease severity, a result which was comparable to treatments of weekly applications of copper-mancozeb.	Lang et al., 2007
Pectobacterium carotovorum ssp. carotovorum	Lettuce	Soft rot	Green house trials showed that phage PP1 could significantly reduce disease development on lettuce plants.	Lim et al., 2013
Streptomyces scabies	Radish	Common scab	Phages Stsc1 and Stsc3 could prevent disease development by treating radish seedlings. Non-treated radishes had 30% less weight than negative control, with phage treated radishes having masses similar to negative control.	Goyer, 2005

(Continued)

TABLE 1 | Continued

Pathogen	Host	Disease	Information	Reference
Xanthomonas axonopodis pv. *citri*	Grapefruit	Asiatic citrus canker	Five greenhouse experiments utilizing phage treatment could reduce disease severity by 59%. However, using a skim milk formulation of phage did not have increased disease control. Phage treatment was also capable of reducing disease occurrence in a citrus nursery. Control was less effective than copper-mancozeb. Combination did not give increased disease control.	Balogh et al., 2008
Xanthomonas axonopodis pv. citrumelo	Orange	Citrus bacterial spot	Phage treatments reduced citrus spot occurrence by 35 and 48% in two trials in commercial citrus nursery. Control was equal or less effective than copper-mancozeb. Combination did not give increased disease control	Balogh et al., 2008
Pseudomonas syringae pv. *porri*	Leek	Bacterial blight	Specific bio-assays demonstrated the *in planta* efficacy of phages vB_PsyM_KIL1, vB_PsyM_KIL2, vB_PsyM_KIL3, and vB_PsyM_KIL3b. However, phage cocktail of six phages (vB_PsyM_KIL1, vB_PsyM_KIL2, vB_PsyM_KIL3, vB_PsyM_KIL4, and vB_PsyM_KIL5 and vB_PsyM_KIL3b), were tested with two parallel field trial experiments in three locations which showed variable results. In one trial, symptom development was attenuated.	Rombouts et al., 2016
Pseudomonas tolaasii	Mushrooms	Brown blotch Disease	Surface of mushrooms were inoculated with pathogen. The formation of blotches was completely blocked by co-incubation of phages with pathogen.	Kim et al., 2011
Erwinia amylovora	Pear, apple trees	Fire blight	Phages ΦEa1337-26 and ΦEa 2345 reduced infection of detached pear tree blossoms by 84 and 96%, respectively, with *Pantoea agglomerans* as a carrier. Also, infection of potted apple tree blossoms could be reduced by 54% with phage ΦEa1337-26 and *P. agglomerans*. Control was comparable to streptomycin.	Boulé et al., 2011

Ideally a phage for biocontrol applications should be exclusively lytic and possess a host range which allows productive infection on all strains of the pathogen genus/species being targeted. Also, current opinion is that phages should be able to lyse the host quickly while producing high numbers of progeny phage and diffuse easily though the environment to which they are being applied. However, there was a report of a phage (ϕRSL1) of the phytopathogen *R. solanacearum* which was described as not highly lytic but still exhibited great biocontrol effect. The current standing theory of this phage's disease prevention ability is that it is capable of co-existing without complete removal of its host from the soil surrounding crop roots, forming an equilibrium of infection that maintains the phage's population but yet suppresses bacteria pathogenicity (Fujiwara et al., 2011).

While a given phage's infection properties may appear to have great potential with *in vitro* studies, this does not necessarily translate into biocontrol potential in the field. Balogh (2006) showed in a study of three phages of *X. citri* pv *citri* exhibiting lytic activity in overlay plate assays that two of these phages were unable to lyse their host bacterium on grapefruit leafs, and indeed were later shown to be ineffective for the suppression of citrus canker in greenhouse trials. Attention should also be paid to the receptors that a given phage recognizes on a bacterial target. This can aid in the creation of phage mixtures with a reduced likelihood of host resistance (Frampton et al., 2014), and as such can lead to the development of phage combinations where individual members work in synergy to eliminate the target bacterium (Born et al., 2011).

ADVANTAGES OF PHAGE BIOCONTROL OVER OTHER STRATEGIES

Unlike chemical biocides, phages occur naturally in the environment and humans are thus exposed to them on a daily basis without any harm. After application, their numbers increase if their target bacterial host species are accessible to them. However, they tend to persist in high numbers in any environment only long as the host is present (Iriarte et al., 2012). Thus, phages are unlike copper-based pesticides which can potentially accumulate in the soil (Hirst et al., 1961; Pietrzak and McPhail, 2004). Phages generally have a narrow host range, typically being limited to stains within a particular species of bacteria. This can allow the creation of phage mixtures which can target bacterial species within a given genus of bacteria only. This could be a specific bacterial phytopathogen or it could be a particular bacterium in a microbial community whose suppression could help improve crop growth. Basit et al. (1992) for example, isolated phage which was unable to infect a desired strain of *Bradyrhizobium japonicum* which could aid soy bean crop growth due its nitrogen fixation properties, but could inhibit competing bacteria which did not

possess this feature, thus allowing enhanced nitrogen fixation to occur.

Biofilm formation is an important factor in the virulence of phytopathogens such a *E. amylovora* (Koczan et al., 2011; Li and Wang, 2014). It is an attribute which has been shown to be involved in bacterial phytopathogen resistance to copper bactericides (Rodrigues et al., 2008). Phages have evolved to overcome this biofilm barrier through the use of depolymerase enzymes on their capsids but can also be released on host lysis, which allows them to degrade biofilm material, allowing the phage anti-receptor to gain access to the receptors on the surface of their host bacterium (Born et al., 2014). There is a growing demand by consumers for food produce that is free from chemicals biocides and preservatives. This has resulted in the restricted use of chemicals to produce "organic label" crops. The requirements of such food require the absence of chemical residues in crop production and processing (Lohr, 2001). Since phages are naturally occurring in the environment, they can be registered as biopesticides, making them suitable for more consumer-friendly organic farming (OmniLytics, 2006).

POTENTIAL ISSUES CONCERNING THE USE OF PHAGE IN BIOCONTROL

The main limitation for the application of phages in biocontrol in most settings is bacterial host-range. While this can be an advantage in certain circumstances, developing a phage-biocide that eliminates every member of a particular bacterial genus or species can be a challenge. Frequently the development of phage mixtures (cocktails) overcomes this disadvantage. Occasionally (but nevertheless, rarely) a phage is isolated which has an unexpectedly broad host-range. One example of this is a phage isolated from sewage and shown to target *Pectobacterium* and also enteric bacteria associated with humans (Pirhonen and Palva, 1988). Thus, careful attention should be given to ensure full understanding of likely host-range of a phage to avoid inefficacy or indeed to avoid the elimination of non-target potentially beneficial bacteria. In the latter context, instances of phage infecting beneficial bacteria resulting in reduced crop yield have been reported (Basit et al., 1992; Ahmad and Morgan, 1994).

It is believed that phages do not directly interact with plants. However, a number of phage-like genes have been identified in wheat, corn and *Arabidopsis* cress (Hedtke et al., 1997; Chang et al., 1999; Ikeda and Gray, 1999) which would suggest incorporation of phage DNA into the genomes of these crops and thus a possible a role in their evolution.

ADVANTAGES OF PHAGES IN THE CONTEXT OF HOST RESISTANCE

Like antibiotics and copper sprays, for which resistance has been reported, there is also the possibility of bacteria becoming resistant to phage infection following constant exposure. However, unlike chemicals, phages are biological entities which can evolve and overcome these biological alterations in their hosts. There has always been a constant race between phage and bacteria in nature. This is indicated by the fact that 10–20% of bacterial populations in certain habitats are lysed daily because of phage infection (Suttle, 1994). In the context of phage resistance, Qiao et al. (2010) found that *Pseudomonas syringae* phage phi2954 was dependent on a host protein glutaredoxin 3 for successful infection. Mutant host strains without this protein were shown to be resistant to the phage. Nevertheless, these authors showed it was possible to isolate mutants of the phage that had become independent of this host protein for infection and this observation has been developed and employed in certain phages aimed at biocontrol. Flaherty et al. (2001) also showed that phages could evolve to overcome phage resistance in target bacteria and these were referred to as H-mutants. This allowed the development of phages with broader host ranges.

In addition to simple mutation-based phage resistance, bacterial phytopathogens can also possess other more complex resistance mechanisms such as the altruistic abortive infection (Abi) systems which give a bacterial host population immunity against a phage by causing phage-infected cells to commit suicide in order to prevent phage reproduction (Parma et al., 1992). While a number of these systems have been identified in *Lactococcus* starter culture strains found in dairy fermentations (Coffey and Ross, 2002; Chopin et al., 2005), recently such a system was identified in the phytopathogen *P. atrosepticum* and was termed ToxIN. This was characterized as a plasmid encoded Type III protein-RNA toxin-antitoxin system. The toxic protein ToxN is bound to RNA antitoxin ToxI in its inactive form. However, when phage infection occured, ToxI RNA antitoxin became unbound from ToxN causing death of the bacterial host cell (Fineran et al., 2009). Indeed, Blower et al. (2012) also showed using phage phiTE, that the phage was capable of creating mutants that could overcome this system by producing a pseudo ToxI RNA antitoxin preventing ToxN toxic activity.

Another mode of phage resistance is CRISPR/Cas systems, which are used by bacteria as well as archaea to form an immunity to protect from infection by foreign DNA such as phage. These systems are comprised of clustered regularly interspaced short palindromic repeat (CRISPR) arrays and CRISPR associated (Cas) proteins. In a recent study of 1,724 bacterial and archaeal genomes it was found that these systems were present in 10% of studied genomes. Previous studies had estimated CRISPR/Cas prevalence values of 40 and 80% of studied bacteria and archaeal genomes, respectively (Burstein et al., 2016). These have been detected in phytopathogens such as *P. atrosepticum* (Przybilski et al., 2011), *E. amylovora* (Rezzonico et al., 2011), and *Xantomonas oryzae* (Semenova et al., 2009). CRISPR arrays are comprised of short stretches of DNA (termed spacers), which are transcribed into short RNAs which interact with Cas proteins to detect and cut foreign DNA that match the sequence of the spacer (protospacer). Spacer sequences are acquired during exposure to foreign DNA in phage or plasmids, and thus they provide a genetic immunity from invasion by foreign DNA due to previous encounters (Marraffini and Sontheimer, 2008). However, it is also possible for phage to evolve to overcome these systems. Indeed, Semenova et al. (2009) detected a spacer in *X. oryzae* which matched a protospacer of phage Xop411. However, the phage was

still able to infect this bacterium, due a mutation having occured in the protospacer sequence.

Bacteria developing resistance against phage infection is not necessarily a negative development in the context of phage biocontrol. Phage-resistance mutations in bacteria frequently are accompanied by a fitness cost, one example being a reduction in virulence, resulting in reduced disease severity. This results from the fact that molecules involved in phage attachment are frequently also involved in the virulence process. Examples include lipopolysaccharide (LPS) (Evans et al., 2010a), extracellular polysaccharide (EPS) (Ayers et al., 1979), flagella (Evans et al., 2010b; Addy et al., 2012) and pili (Ahern et al., 2014). Thus, mutations leading to resistance frequently compromise virulence. There, are however, a few examples where these mutations in bacteria surface structures did not lead to reduced virulence as seen with LPS production mutants of *Pectobacterium* and *Dickeya* (Schoonejans et al., 1987; Pirhonen et al., 1988).

BACTERIOPHAGE AND CHEMICALS

Phage have been shown to be stable in certain agrichemicals (Ravensdale et al., 2010). However, precautions need to be taken with some chemicals being combined with phage. Chemical biocides typically contain a range of phage inactivating substances such as surfactants and chelators (Yamamoto et al., 1968; Chattopadhyay et al., 2002). Also, copper-based bacteriocides have been shown to inactivate phage, but this inactivation can be avoided with the delayed application of phage (4–7 days) after initial application of copper-based bactericide (Iriarte et al., 2007).

COMPLEXITY OF PHAGE INTERACTION WITH SOIL

The rhizosphere is the area of soil which is in close proximity to the roots of a plant. There are several factors which can affect phage activity in this environment such as pH, moisture levels, presence of organic matter and soil type. A number of these factors either individually or in combination can cause phage inactivation. Different soil types affect the survival of phage. For example, clay loam soils appear better at maintaining phage at low soil moisture levels and high soil temperatures than that of sandy loam soils (Straub et al., 1992) As well, low soil pH can also negativity affect phage survivability (Sykes et al., 1981).

Levels of adsorption of phage are affected differently in differing soil types, with levels of hindrance varying from one phage type to another (Goyal and Gerba, 1979). Phage can become bound to soil components such as clays (kaolinite and montmorillonite) as these minerals possess positively and negatively charged surfaces to which phage can adsorb (Schiffenbauer and Stotzky, 1982). Such adsorption can be influenced by pH (Goyal and Gerba, 1979; Loveland et al., 1996) as well as the presence of organic materials (Zhuang and Jin, 2003). Under favorable conditions, phages have been identified that persist at relatively stable concentrations for several weeks in soil (Fujiwara et al., 2011).

PHAGE IN THE PHYLLOSPHERE

The phyllosphere is the portion of the plant which is above the ground and phages can readily be isolated from this location. How phages get there naturally has not been defined precisely, although it is possible that they originate in the soil from which the plant germinated - or alternatively get deposited by insect vectors. Indeed, phages for the phytopathogens *Pantoea stewartii* and *Erwinia herbicola* var. *herbicola* have been isolated from corn flea beetles (Woods et al., 1981). Another route is the translocation of phage from the roots to leaves of plants through the plant vascular system. And it has been shown that phages of *R. solanacearum*, *Xanthomonas perforans*, and *Xanthomonas euvesicatoria* can translocate though tomatoes plants, phage of *Xanthomanas oryzae* though the rice seedlings and phages of *E. amylovora* though apple seedlings and fire thorn (Rao and Srivastava, 1973; Iriarte et al., 2012; Kolozsváriné Nagy et al., 2015). However, this translocation may be influenced by the phage type, plant age, plant size, plant species, plant health and possibly soil type in which the plant is growing (Iriarte et al., 2012). It has also been reported that *E. amylovora* phages could pass from the leaves to the roots of apple seedlings (Kolozsváriné Nagy et al., 2015). The phyllosphere is nevertheless a harsh environment for phages to survive and it has been reported that their numbers can rapidly decline during daylight hours (Balogh et al., 2003; Iriarte et al., 2007). The destructive influence of UV light from the sun has been reported to be a limiting factor for the application of phages for successful biocontrol. The radiation causes the formation of lesions in DNA which can block DNA replication and transcription. In an vivo study with phage phiXV3-16, Iriarte et al. (2007) demonstrated a direct relationship between phage reduction on tomato leaves and increasing UVA+B dose. They also showed in an *in vitro* study that UV was capable of inactivating phage used against *Xanthomonas campestris pv. vesicatoria,* preventing it from exerting a biocontrol effect. Phage sensitivity against UV light has been shown to occur also with phage of *Dickya solani and E. amylovora* phages (Czajkowski et al., 2014; Born et al., 2015). However, there have been phages isolated against the phytopathogen *Pseudomonas syringae pv. actinidiae* which can tolerate extended UV-B doses (Yu et al., 2015). Other potential factors that could cause phage decline on the phyllosphere are desiccation, temperature, pH as well as certain chemicals produced by plants (Erskine, 1973; Delitheos et al., 1997; Iriarte et al., 2007).

PHAGE APPLICATION METHODS FOR OPTIMAL BIOCONTROL PERFORMANCE ON PLANTS

One of the limitations to effective phage biocontrol on crops is the possibility of poor persistence on the phyllosphere due to

the factors discussed in the previous section. However, several methods have been found to reduce this problem. Survival of phage can be improved in the phyllosphere and rhizosphere if they are accompanied by a viable host. This can be an avirulent strain of the pathogen being targeted or indeed another species of bacteria which occurs naturally in that environment (Svircev et al., 2006; Bae et al., 2012; Iriarte et al., 2012). It has also been found that avoiding daylight during application can improve phage-based biocontrol. Indeed, it has been demonstrated that applying phage to tomato leaves in the evening resulted in longer phage persistence in the phyllosphere, giving phage more time to infect and kill their bacterial targets (Balogh et al., 2003; Iriarte et al., 2007).

Born et al. (2015) conducted studies with number of substances to investigate if they gave phage protection against UV and reported that natural extracts from carrot, red pepper and beetroot all gave protection as did casein, soy peptone and also purfied aromatic amino acids, astaxathin and Tween 80. None of these subtances had a compromising effect on phage infection and stability (Born et al., 2015). Thus, it appears that a wide range of substances could enhance phage preformance in the phyllosphere with the main requirement being that they need to absorb UV thus limiting phage exposure. Biodegradable polymers have also been shown to give these protective effects (Khalil et al., 2016). In addition, Balogh et al. (2003) also showed an enhanced phage activity by combining the following preparations with phage, namely (i) 0.5% pregelatinized corn (PCF) and 0.5% sucrose, (ii) 0.5% Casecrete NH400, 0.5% sucrose and 0.25% PCF and (iii) 0.75% skim milk and 0.5% sucrose. These tests were performed in greenhouse trials and in field trials on tomato plants with phages against *Xanthomonas campestris* pv. *vesicatoria.* All formulations were used under a variety of different conditions, but generally demonstrated enhanced disease protection.

Soil based phage delivery is another approach that has been looked at to improve phage presistance in the phylosphere. Iriarte et al showed that a proprietary mixture of phage (OmniLytics Inc.) active against *X. perforans* strain 97-2 could translocate to the upper leaves of a tomato plant from its roots. They demonstrated that these phages which were applied to soil at levels of 10^8PFU/mL could be detected at titres of 10^4 PFU/g in leaf tissue for 7 days, whereas with a direct foliar application of the same phage mix, phage were undetectable 1 to 2 days after application (Iriarte et al., 2012). This work would suggest that the phage control of foliar plant diseases could be controlled by applying the phages to surrounding soil of a plant rather than by foliar spraying.

COMBINATION OF PROTECTIVE METHODS APPEAR TO BE THE BEST DIRECTION FOR PHAGE BIOCONTROL

There is evidence to support that combining phage with several methods used to control crop disease results in better control. The bacterium *Pantoea agglomerans* has been used as a biocontrol agent to suppress growth of the agent of fire blight *E. amylovora* and is being sold under the band name Bloomtime® (Mikiciński et al., 2016). However, it has been reported that combining this bacterium with phage biocontrol can give enhanced protection that is comparable to that achieved with the antibiotic streptomycin (Svircev et al., 2006; Boulé et al., 2011). A similar observation of enhanced control was seen using a bacteriocin-producing strain of *R. solanacearum* with a phage to combat tobacco bacterial wilt (Tanaka et al., 1990). In another study, combining phage with Acibenzolar-S-methyl (ASM) was shown to have improved protection against bacterial spot of tomato in the field (Obradovic et al., 2004). However, combinations of phage with copper based pesticides do not appear to produce synergistic effects. Treatment with copper-mancozeb as seen with citrus canker and bacterial spot of citrus fruits did not produce synergy against *Xanthomanas axonopodis* pv.*citri* or *Xanthomanas axonopodis* pv. *citrumelo,* respectively (Balogh et al., 2008). As mentioned previously, this could be due to phage sensitivity to the components of these copper based sprays.

IMPROVED UNDERSTANDING OF BACTERIAL HOST DIVERSITY SHOULD AID PHAGE BIOCONTROL AND IMPROVE ITS SUCCESS IN THE FUTURE

Recent years have seen recognition of the increasing diversity and complexity of bacterial phytopathogens mainly due to advances in molecular techniques (16S rRNA sequencing). For example, *X. campestris* pv. *vesicatoria,* which was previously a single species has since been divided into four (Jones et al., 2004). Another example is of the soft rot *Erwinia* group, which has undergone a significant taxonomic reshuffle with creation of novel species and genera (Hauben et al., 1998; Gardan, 2003; Samson et al., 2005). These developments are very important, as while the afflictions caused by these bacteria may appear identical on their respective crop targets, the phage sensitivities of the pathogens are likely to differ significantly, but nevertheless are likely to have some correlation with their taxonomic groupings. For example, the soft rot *Erwinia* group, which affects potato crops, has more recently been reclassified into two new bacterial genera (*Pectobacterium* and *Dickeya*), and these are relatively distinct from the point of view of phage susceptibilities (Czajkowski, 2016).

PHYTOPATHOGENS TARGETED FOR PHAGE BIOCONTROL AND HOW THEY ARE CURRENTLY MANAGED

There are a number of important bacterial plant pathogens that have received attention for phage biocontrol in recent years (**Table 1**) as existing approaches are having limited efficacy or their use is restricted in certain regions of the world. The following section discusses selected crop pathogens where phage biocontrol has been evaluated and is showing promise.

Dickeya and *Pectobacterium*

Both *Dickeya* and *Pectobacterium* belong to the family of *Enterobacteriacea*, which collectively can be referred to as the Soft Rot *Enterobacteriacea* (SRE). Both genera characteristically produce several cell-wall-degrading enzymes that allow them to infiltrate and macerate the plant tissue on which they feed (Pérombelon, 2002). The plant host range of both bacterial genera is very broad: species belonging to *Dickeya* have been reported to infect 10 monocot and 11 dicot families, while those of *Pectobacterium* are reported to infect eleven monocot and sixteen dicot families (Ma et al., 2007).

P. carotovorum ssp *carotovorum* has a wide host range and global distribution, while *P. atrosepticum* is primarily found in temperate climates with a host range mainly limited to the potato (Pérombelon, 2002). *P. wasabie* and *P. carotovurum* ssp. *brasilensis* are also found to infect potato in several regions worldwide (Waleron et al., 2013; Lee et al., 2014). In Europe, *Dickeya dianthicola* is reported to be very important in potato disease, although more recently, a new *Dickeya* species called *D. solani* is being more frequently identified. Both also cause disease in other regions of the world (Toth et al., 2011). The economic impact of these potato infections can be severe. In the Netherlands, they cause annual losses in the seed potato sector of as much €30 million per year and in Israel, potato yield losses due to *Dickeya* have been as much as 20–25% (Prins and Breukers, 2008; Tsror (Lahkim) et al., 2008).

With regard to the potato, there are no effective bactericides to protect against SRE and the most effective approach has been through careful culturing practices, involving avoidance of contamination and the removal of diseased plants and/or diseased tissue. Certification systems are also employed. These involve the propagation of seed plants using healthy tissue culture plantlets followed by propagation in greenhouses, and then open field grow-out production. It is accompanied by careful monitoring and removal of diseased plants before release for general production. The generation number of these crops is also kept low to limit bacterial build up. However, the success of these certification schemes has been variable and heavily weather dependant (De Boer, 2004; Czajkowski et al., 2011).

Erwinia amylovora

Erwinia amylovora, a member of the family of *Enterobacteriacea,* is the causative agent of fire blight which is a destructive disease that occurs to species of the plant family *Rosaceae.* The disease has been reported in 40 countries across North America, Europe, the Pacific Rim, and the Middle East (Bonn and van der Zwet, 2000). It heavily affects apple and pear production in several regions, with costs estimated as much as $100 million per year in the USA due to production losses and control measures (Norelli et al., 2003). It is considered to be a quarantine concern in countries belonging to plant protection agencies of APPPC (Asia and Pacific Plant Protection Commission), COSAVE (Comite Regional de Sanidad Vegetal para el Cono Sur), EPPO (Europe and Mediterranean Plant Protection Organisation) and IAPC (Inter-African Phytosanitary Council) (CABI, 2016).

Pathogenesis typically involves the bacterium entering a susceptible plant host though the nectarthodes of its flowers, but it may also enter the plant though other openings such as wounds (Bubán and Orosz-Kovács, 2003). Once in the plant, it is capable of moving though the intracellular space of parenchyma, where at the latter stages it may reach the xylem vessels. Under favorable conditions, disease can present itself as wilting, necrosis of tissue and dieback of the plant (Vanneste and Eden-Green, 2000). The bacterium does not produce cell-wall-degrading enzymes but the exopolysaccharide amylovoran, biofim formation capacity, motility, a type III secretion system, and quorum sensing are all understood to be features in its virulence (Piqué et al., 2015).

Traditionally, control of fire blight relies on cultural practices involving the removal of diseased tissue as well as preventative sprays containing copper or antibiotics (Norelli et al., 2003). However, issues with these chemical controls is copper tolerance of the pathogen and also the long term of use antibiotics (such as streptomycin) as a control strategy may be limited in the future, with growing concern of antibiotic resistance and the resulting restricted used of antibiotics for agriculture in certain regions of the world such as EU countries (Ordax et al., 2006; Russo et al., 2008; Mayerhofer et al., 2009; de León Door et al., 2013). As mentioned, biological controls using antagonistic bacteria have shown a capacity for controlling the disease (Mikiciński et al., 2016)

Ralstonia solanacearum

Ralstonia solanacearum is a Gram negative soil-borne bacterium. It is considered to be one of most destructive phytopathogens with a host range of up to 200 plant species from over 50 families (Denny, 2007). The bacterium is highly heterogeneous, historically being divided into five races (based on plant host range) and five biovars (based on carbon utilization) (Denny, 2007). It causes diseases of economically important crops, such as bacterial wilt of tobacco, banana and tomato as well as brown rot of the potato (Sanchez Perez et al., 2008). The bacterium has global distribution (Sanchez Perez et al., 2008), and with regard to tomato and potato production, has quarantine status in the EU (Anonymous, 2000). The species has considerable economic impact: for example, brown rot of the potato has been estimated to exceed more than €950 million in losses per year worldwide (Scherf et al., 2010). Infection begins by the bacterium entering the host plant though its roots where it will then colonize the xylem. Infection typically leads to the development of yellowing of the plant, stunted growth, wilting and death, although the bacterium is also capable of asymptomatic infections (Sanchez Perez et al., 2008). Typical methods of control include the use of cultural practices such as selection of planting time, crop rotation, using clean seedlings and the use of resistant cultivars (Mariano et al., 1998). However, the use of such cultivars has shown a negative correlation between resistance and yields (Yuliar et al., 2015). Also, resistance possessed by these cultivars tends to be strain specific (Wang et al., 2000).

Pseudomonas syringe

The bacterial phytopathogen *P. syringea* belongs to the class of *Gammaproteobacteria* (Hirano and Upper, 2000). The species is

currently subdivided into more than 50 pathovars, with different pathovars representing different strains with differing plant host ranges (Hirano and Upper, 1990; Parkinson et al., 2011). Stains of most pathovars typically exhibit narrow host ranges, with pathovar P. *syringea* pv. *syringea* being an exception, having been reported to infect more than 80 plant species (Hirano and Upper, 2000).

Pseudomonas syringea pv. *tomato* causes necrotic lesions surrounded by a yellow chlorotic halos on tomato, a disease known as bacterial speck (Cruz et al., 2010). The pathovar can also infect members of genera of *Arabidopsis* and *Brassica* in laboratory setting (Elizabeth and Bender, 2007). The disease reduces yields while also affecting fruit quality (Fatmi, 2003). Pathogenesis by the bacterium involves the invasion of plant tissue from natural openings, such as stomata, where a type III secretion system plays a major role in its virulence with the release of effectors to overcome the plant immune system (Xin and He, 2013). It is spread by contaminated tomato seeds but can also survive as an epiphyte for extended periods on tomato plant surfaces and is dispersed in windblown rain (Smitley and McCarter, 1982; McCarter, 1983; Preston, 2000). Control of the organism typically involves the use of uncontaminated seeds and the used of bactericides (copper and streptomycin) to limit its spread (Preston, 2000; Fatmi, 2003). However, copper tolerant strains of the bacterium have been reported (Alexander et al., 1999).

Xanthomonas species

Xathomonas is a large genus, which belongs to the class of *Gammaproteobacteria*, containing at least 27 official species, many of which also possess several pathovars. Collectively, the genus host range is broad: infecting around 400 plant hosts, a number of which are important crops such rice, banana, tomato, and citrus fruits. Species and pathovars of this genus typically exhibit a high degree of host- as well as tissue-specificity, invading either the xylem or intercellular spaces of the mesophyll parenchyma tissue (Ryan et al., 2011).

Xathomonas campestris pv. *vesicatoria* is the causitive agent of bacterial spot disease of tomato and pepper, with the disease having been identified in many countries worldwide (Jones et al., 2005). This tomato disease can be very severe with yield losses of up to 50% reported for tomatoes grown both in greenhouses and fields in the USA and Caribbean (Camesano, 2015). Disease is caused by the bacterium entering the plant though stomata or wounds. The bacteria then colonize the intercellular space of the plant, inducing water-soaked lesions that later become necrotic, which can result in defoliation and severely spotted fruit (Thieme et al., 2005). Control of the disease has involved preventative cultural practices such as avoiding unnecessary crop damage and using uncontaminated seed, but also includes use of resistant cultivars as well as chemical controls with copper or streptomycin (Goode and Sasser, 1980). However, the use of resistant cultivars has not always been successful and there have been reports of bacteria developing resistance to the above two agents (Goode and Sasser, 1980; Ritchie and Dittapongpitch, 1991; McDonald and Linde, 2002).

Xylella fastidiosa

Xylella fastidiosa belongs to the of class of *Gammaproteobacteria.* It is a xylem-limited phytopathogen that requires insect vectors (such as sharpshooters) for its distribution and infection of its host plants (Chatterjee et al., 2008). It causes disease on a number of crops such as the grape, citrus, almond, peach and coffee (Hopkins and Purcell, 2002). While it has primarily been contained in the Americas, it has been indentified in Europe in recent years causing disease on olive trees (Hopkins and Purcell, 2002; Loconsole et al., 2014). Disease caused by the bacterium is believed to be induced by the formation of biofilm aggregates in the vascular system, which restricts the movement of nutrients and water throughout the plant (Chatterjee et al., 2008). It causes Pierce disease of the grapevine, a highly destructive infection, which heavily affects grape production in the USA, and has been estimated to cost as much as $104.4 million annually to the state of California (Tumber et al., 2014). Existing control methods have been limited in their management of the disease and include removal of infected plants and control of the infected insect vector populations with neonicotinoid-based insecticides (Janse and Obradovic, 2010). However, the use of these insecticides has seen restrictions in recent years due to their possible effects on honey bee populations (Anonymous, 2013; Lu et al., 2014).

CRITICAL SUMMARY OF RECENT PHAGE BIOCONTROL STUDIES ON CROPS

There is growing evidence showing that phage have promising biocontrol applications for number of plant diseases in different crops. The following section describes recent studies that have been conducted since the year 2000 and the findings from these is summarized in **Table 1**.

The most common crops that appear to benefit from the application of phages for biocontrol in recent scientific literature are the potato and the tomato, as both have been the focus of numerous recent studies. The bacterial pathogens in the case of the potato are predominantly the SRE. As mentioned above, one of the most important SREs in Europe is *D. solani;* and the potential of phage to control this phytopathogen have been assessed indicating strong potential for disease control. For example, Adriaenssens et al. (2012) conducted a bioassay and a field trial using phage (LIMEstone1). The bioassay involved the incubation of seed tubers (cultivar Bintje), which had either been inoculated with the bacteria or co-inoculated with the bacteria and the phage (MOI of 100). They showed that tubers inoculated with the bacteria alone would experience to 40% maceration of tuber tissue, while those co-inoculated with the phage and bacteria exhibited no more than 10% maceration of tuber tissue. Similar results were observed with the seed tuber cultivar Kondor. The field trial using the same phage against the same pathogen also suggested it was capable of exerting this biocontrol effect *in-planta,* as phage treated infected seed potatoes resulted in higher crop yields than those without

phage treatment. Similar findings were reported by Czajkowski et al. (2014) who also isolated phages specific for *D. solani*. These workers conducted bioassays with tuber slices incubated with the bacterial pathogen with or without phages (MOI of 0.01) and showed that the application of phages could prevent potato tuber tissue maceration by up to 70%. SREs other than *D. solani* were also studied for their susceptibility to phages by the same group. They found that the application of phages (MOI of 0.01) to control *P. carotovorum ssp carotovorum* and *P. wasabie* destruction could prevent damage of up to 80% on tuber slices and up to 95% on whole tubers against tissue maceration from a mixed bacterial infection (Czajkowski et al., 2015). Such data is highly encouraging as many SRE infections tend to result for a mixture of genera/species. Aside from potato, SRE infections have also been controlled by phage in lettuce, with high levels of disease prevention being reported (Lim et al., 2013). Aside from the SRE problem, potato infections from the Gram-positive bacterium *Streptomyces scabies* results in the formation of a corky lesion (known as common scab) on the tuber and indeed other root vegetables also, as well as causing the reduced growth of seedlings (Lerat et al., 2009). This pathogen has also been successfully treated in potato by phage biocontrol and thus has implications for other crops also as demonstrated by Goyer (2005). In conclusion, the above studies indicate strong potential for phage based control of these diseases.

Another crop which has been the focus of several studies in the context of phage therapy is the tomato, which is commonly infected by *R. solanacearum* (also causes brown rot in the potato) and *X. campestris* pathovars. Again, phage biocontrol approaches have been demonstrated to give a significant reduction in bacterial wilt (*Ralstonia*) and leaf spot caused by *Xanthomonas*. Indeed, the successful trials against *R. solanacearum* reported by Mansfield et al. (2012) are significant considering the wide host range of the bacterium. Similarly, in the case of *Xanthomonas*, the observed beneficial effect of the application of phages can also be extrapolated to other plants affected by pathogens belonging to the same genus. Indeed, studies on elimination of *Xanthomonas* using phage have been conducted with successful outcomes on both grapefruit and orange (Balogh et al., 2008) as well as onion (Lang et al., 2007). A variety of other crop infections have also been reduced in severity in other phage biocontrol studies. These include *Pseudomonas* infections of mushrooms (brown blotch) and leeks (bacterial blight) and infection of the grapevine by *Xylella* (Das et al., 2015).

COMMERCIALIZATION OF PHAGE FOR BIOCONTROL IN CROP DISEASE

In recent years, several phage biocontrol products have reached the market. A USA based company Omnilytics was the first company to receive registration (from the US Environmental protection agency) for their phage based biopesticide product Agriphage. The product is designed for the control of bacterial spot or speck of tomatoes and peppers (specific for *X. campestris* pv. *vesicatoria*, or *P. syringae* pv. *tomato*). This product has also received an OMRI listing making it suitable for use by commercial organic growers (OmniLytics, 2006). A Hungarian company Enviroinvest was the second company to receive registration for their biopesticide named Erwiphage for the control of fire blight of apple trees (specific for *Erwinia amylovora*) (Enviroinvest, n.d.). There is also a Scottish company, APS biocontrol, which has developed a bacteriophage-based wash solution (Biolyse) for potatoes tubers, which is to be used for prevention of soft rot disease (specific against soft rot *Enterobacteriacea*) during storage (APS Biocontrol Ltd, n.d.). Interestingly this product has been reported to be used by the Tesco supermarket chain (Branston, 2012).

However, in some regions of the world there are delays that have to be overcome with regard to legislation allowing phage biocontrol approaches for the of control of bacterial plant diseases. A problem with phage-mediated biocontrol is that phage mixtures/cocktails need to be updated constantly in order to lyse as many newly emerging strains of the target bacterium as possible. This approach is used by Omnilytics (OmniLytics, 2004). This allows a phage cocktail to be adapted to the relevant disease-causing bacterial strains in a given situation, also facilitating counteraction of any phage resistance development during the phage application. However, EU regulations (1107/2009 EC) require that any change to one of the components of a phage cocktail would require reregistering which requires time and expense, making the US approach currently unfeasible in the EU (Doffkay et al., 2015). Legislation governing phage biocontrol may need to become more malleable in the EU for the best application and performance of phage products as biopesticides.

OTHER PHAGE APPLICATIONS OF THE PAST AND POSSIBLE FUTURE WITH REGARDS TO PHYTOPATHOGENS

Phage typing schemes have been employed for several phytopathogens for epidemiology studies (Toth et al., 1999; Ahmad et al., 2014). These systems allow the identification of a particular strain of a species based on their susceptibility to series of phage. The downfall of this method, however, is that it depends on the isolation of pure cultures for identification as well as the maintenance of stocks of typing phage as well as host strains for which to propagate them. Nowadays, studies of phytopathogens has moved away from phage typing due to its tendency to generate false positives and false negatives results as well as its low resolution and the development of new and improved molecular techniques (Czajkowski, 2016)

Several phage-based detection systems have been developed for human and animal pathogens (van der Merwe et al., 2014). Recently however, work has been published on the promising application of these methods for the detection of plant pathogens. Such a detection system has been developed for *R. solanacearum,* which is based on detection of the bacterium by phage propagation followed with quantitative PCR (qPCR). Samples that contain the bacterium will cause added phage

titres to increase, these titre increases can then be detected using qPCR. This method was found to be faster than conventional methods with greater sensitivity allowing detection of 10^2 CFU/g of soil, 10^3 CFU/ml from drainage water from potted plants and 10^2 CFU/g in 0.1 g of leaf tissue. The method also does not require the destruction of a plant for the detection of bacterium unlike those currently used to detect *R. solanacearum* (Kutin et al., 2009). It is possible to engineer phage of phytobacteria into reporter systems that can emit a detectable bioluminescent signal during infection. A "*luxAB*-tagged" reporter phage was developed for *Pseudomonas cannabina pv. alisalensis* (agent of bacterial blight of crucifers) which was shown capable of detecting the bacteria within minutes. This phage was also capable of emitting a detectable signal during infection of both cultures and diseased plant samples (Schofield et al., 2013). Both the mentioned systems have advantages over other molecular detection methods, in that phage propagation requires active metabolism, conveniently limiting it to viable bacterial cells.

CONCLUSION

Effective control of plant disease typically calls for a disease management strategy that involves several integrated approaches. Currently, the use of phage biocontrol is an emerging, but as yet uncommon practice. However, phages do possess several properties which can add to the arsenal of controls for crop diseases. They are natural, making them suitable for organic farming. They can be used to create phage cocktails with tailored host ranges. Also, phages naturally have the potential to evolve to adapt to overcome phage-resistance or overcome new strains of bacteria. They can be combined with other chemical or biocontrol agents. A possible limitation to their use is their sensitivity to UV light and to certain soil conditions. However, approaches have been found to overcome some of these limitations with the use of UV protectant formulas and timing of the application of phage to crops to avoid interaction with chemical pesticides and exposure to UV light. In addition to biocontrol applications, there is also good potential for phage-based diagnostics for plant pathogenic bacteria with a high sensitivity aimed specifically at viable bacteria.

Many pesticide companies are moving away from investment in chemical pesticides and increasingly directing their attention to biopesticides. The pesticide market is worth $56 billion with the biopesticides forming only $2–3 billion of this. However, growth of the biopesticide sector is expected to outpace chemical pesticides in the future (Marrone, 2014). This change is believed to be due to an increasing customer demand for chemical residue free foods and increasing legalization on the use of synthetic pesticides in certain regions in the word. In addition, many biopesticide products are potentially cheaper to develop and quicker to bring to the market (Marrone, 2014). With this economic environment, one can expect to see increased activity in the development of phage biocontrol as a viable approach for crop disease control in the future.

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REVIEW
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Adapting Drug Approval Pathways for Bacteriophage-Based Therapeutics

*Callum J. Cooper *, Mohammadali Khan Mirzaei † and Anders S. Nilsson **

Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

The global rise of multi-drug resistant bacteria has resulted in the notion that an "antibiotic apocalypse" is fast approaching. This has led to a number of well publicized calls for global funding initiatives to develop new antibacterial agents. The long clinical history of phage therapy in Eastern Europe, combined with more recent *in vitro* and *in vivo* success, demonstrates the potential for whole phage or phage based antibacterial agents. To date, no whole phage or phage derived products are approved for human therapeutic use in the EU or USA. There are at least three reasons for this: (i) phages possess different biological, physical, and pharmacological properties compared to conventional antibiotics. Phages need to replicate in order to achieve a viable antibacterial effect, resulting in complex pharmacodynamics/pharmacokinetics. (ii) The specificity of individual phages requires multiple phages to treat single species infections, often as part of complex cocktails. (iii) The current approval process for antibacterial agents has evolved with the development of chemically based drugs at its core, and is not suitable for phages. Due to similarities with conventional antibiotics, phage derived products such as endolysins are suitable for approval under current processes as biological therapeutic proteins. These criteria render the approval of phages for clinical use theoretically possible but not economically viable. In this review, pitfalls of the current approval process will be discussed for whole phage and phage derived products, in addition to the utilization of alternative approval pathways including adaptive licensing and "Right to try" legislation.

Keywords: bacteriophage, phage therapy, adaptive pathways, alternative licensing, pharmaceutical regulation

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University College London, UK

Reviewed by:
Eric Christopher Keen,
Washington University in St. Louis, USA
Diana R. Alves,
Blond McIndoe Research Foundation, UK

****Correspondence:***
Callum J. Cooper
callum.cooper@su.se
Anders S. Nilsson
anders.s.nilsson@su.se

†Present Address:
Mohammadali Khan Mirzaei, Department of Microbiology and Immunology, McGill University, Montreal, QC, Canada

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INTRODUCTION

The discovery of penicillin in 1928 heralded a dynamic shift in modern medicine with antibiotics quickly becoming one of the linchpins of modern medicine (Zaffiri et al., 2012). However, in the 1960s, the "golden era" of the identification of novel antibiotics ended with modern development focusing on the modification of existing drugs (Nathan and Cars, 2014) with only four multinational pharma companies maintaining antibiotic divisions (Fair and Tor, 2014). This lack of interest is not only due to the difficulties in discovering new antibiotic classes but also decreasing financial returns within drug development (Scannell et al., 2012). It is particularly true for anti-infective agents, where a median 10 day drug-treatment costs ∼US$ 85 for non-HIV anti-microbial drugs compared to ∼US$ 848 for anti-neoplastic drugs (Falagas et al., 2006). When compared to the total number of drugs granted regulatory approval, anti-infective drugs represent due to a poor return on overall investment which stifles their development (**Figure 1**; Piddock, 2012).

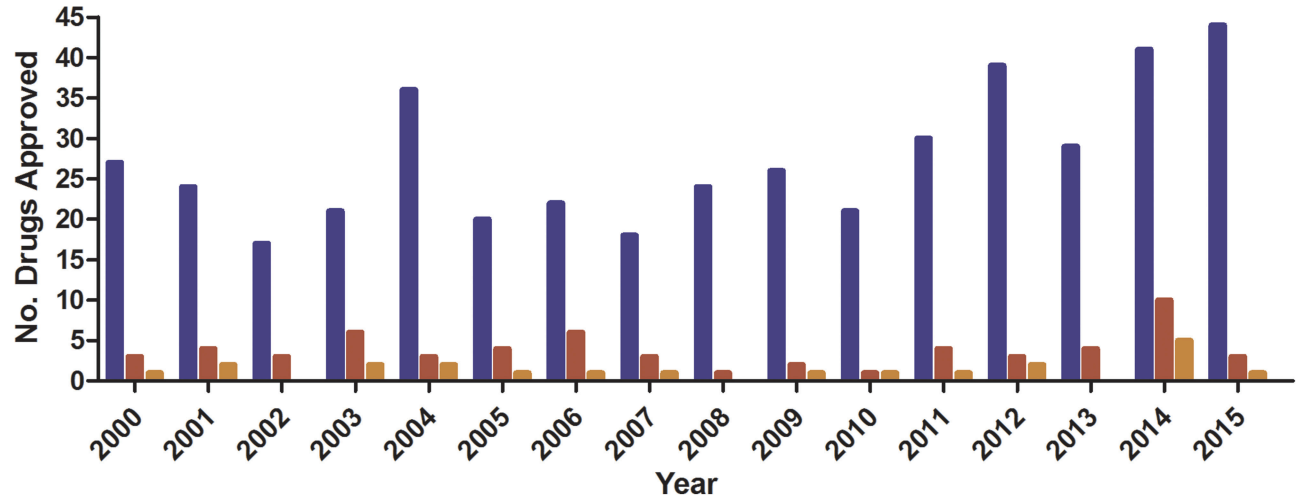

FIGURE 1 | FDA Novel Drug Approval 2011–2015. Data obtained from (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugInnovation/ucm430302.htm). ■, Total; ■, anti-infective; ■, anti-bacterial.

Despite public concern about increasing levels of antibiotic resistance, antibiotic consumption continues to increase, particularly in the BRIC (Brazil, Russia, India, China) nations (Van Boeckel et al., 2014). The ability to obtain antibiotics without prescription, their subsequent misuse by patients (Li, 2014), and the continued use of antibiotics as a growth promoter in agriculture (Cully, 2014), has contributed to an increase in the number and scale of multi-drug resistant infections (Molton et al., 2013). Increased consumption and fervid media reporting has generated huge interest in the development of new antibacterial agents and has led to the formation of a number of working groups, such as the US Food and Drug Administration's (FDA) Antibacterial Drug Development Task Force (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/ucm317207.htm; accessed 13th June 2016) or the Biotechs from Europe innovating in Anti-Microbial resistance (BEAM) Alliance (http://beam-alliance.eu/; accessed 13th June 2016). These groups have called for financial incentives and patent extensions to be applied to antibacterial drug development in order to stimulate research (Sonderholm, 2009; Laxminarayan and Powers, 2011; Wise, 2015).

Interest in phage therapy (i.e., the clinical use of bacteriophage based therapeutic products in humans), has been traditionally confined to academic groups and a few clinical centers in Eastern Europe, most notably the ELIAVA Institute in Tbilisi, Republic of Georgia. However, the specificity of phages and the enormous variation in human—bacteria—phage combinations will lead to an immense number of obligatory clinical trials if they are to be considered as a viable alternative to antibiotics. This constitutes one of the primary obstacles for the industrial development of phage based therapeutic products, in addition to concerns over intellectual property protection. Nevertheless, commercial interest has been piqued in the form of small BioPharma companies (**Table 1**), but significant interest from multinational pharmaceuticals is still lacking. These small BioPharma companies have enabled a number of commercial phage products to be approved for use in reducing food contaminants (Endersen et al., 2014), but widespread use of phage therapeutics in humans remains elusive in the West (Kingwell, 2015).

The societal need for new antibacterial agents, and the knowledge that phage therapy may work in practice, requires the engagement of commercial entities to further develop phage based products rather than proceeding as a purely academic enterprise. However, what appears to limit the development of phage products for human use is primarily associated with development costs and regulations. Through the application of new or refined regulations the development of phage based pharmaceutical products may become faster and more commercially attractive for companies.

In this article, the current requirements for the development and approval of new antibacterial drugs are described with emphasis placed on the challenges faced by phages and phage based products. Potential alternative or additional approval pathways within existing and proposed legislation and how phage therapy could benefit from these pathways are also discussed.

THE REVIVAL OF PHAGE THERAPY

Since the early part of the 20th Century, bacteriophages have been used to treat a range of different bacterial infections (Kutter et al., 2010). However, since the introduction and success of antibiotics in the mid-20th century, interest in phages as antimicrobial agents within Western Europe and the US has waned. Increasing problems with antibiotic resistant bacterial infections has led to alternative strategies being sought. This has in turn revitalized research into bacteriophages and their derived products as antibacterial agents (Oliveira et al., 2015). Phage therapy possesses advantages and disadvantages when compared to conventional antibiotics. These advantages include the ability

TABLE 1 | Summary of current phage based products in development for the treatment of human disease.

Company	Product	Type	Target	Application	Company website
Micreos	Staphefekt	Endolysin	*S. aureus*	Topical	https://www.staphefekt.com/en/newspublications
Intralytix	ShigActive	Phage	*Shigella*	Ingested	http://www.intralytix.com/index.php?page=prod
AmpliPhi	AmpliPhage-001	Phage	*Ps. aeruginosa*	–[a]	http://www.ampliphibio.com/product-pipeline.html
	AmpliPhage-002		*S. aureus*	Topical	
	AmpliPhage-004		*C. difficile*	–	
Technophage	TP-102	Phage	–[a]	Ulcers	http://www.technophage.pt/index.php/r-d/product-pipeline
	TP-122		–[a]	Respiratory	
	TP-132		–[a]	–[a]	
	TP-107		–[a]	Topical	
Pherecydes Pharma	PP021	Phage	*E. coli*	Burn and Skin	http://www.pherecydes-pharma.com/pipeline.html
	PP1131PP1231		*Pseudomonas*	Burn, Skin, and Respiratory tract infection	
	PP2351		*Staphylococcus*	Bone, Joint, and Prosthesis	
Avid Biotics	Pyocin	Phage Derived	*E. coli*	Diarrheal and food poisoning	http://www.avidbiotics.com/programs/
	Avidocin		*C. difficile*	–[a]	
	Pyocin		*Pseudomonas*	–[a]	
	Purocin		*Salmonella*	Food poisoning	
	Purocin		*Listeria*	Food poisoning	
ContraFect	CF-301	Phage Derived Lysins	*S. aureus*	–[a]	http://www.contrafect.com/pipeline/overview
	CF-303		*S. pneumoniae*	–[a]	
	CF-304		*S. faecalis* and *E. faecium*	–[a]	
	CF-305		*S. agalactiae*	–[a]	
	CF-306		*B. anthracis*	–[a]	
	CF-307		Group B Streptococcus	–[a]	

[a]*Information not available.*

of phages to self-replicate in the presence of a suitable bacterial host. They act with minimal disruption to the local microbiota and are relatively easy to isolate from environmental sources, while the limited host range of lytic phages may detract from their overall clinical usefulness. Although the advantages and disadvantages of phage therapy have been briefly highlighted here, they are discussed extensively elsewhere (Loc-Carrillo and Abedon, 2011; Nilsson, 2014; Kutter et al., 2015).

Virulent phages have been isolated from a variety of environments and proven *in vitro* to be efficient against a large number of bacterial species (Mattila et al., 2015; Salem et al., 2015; Sauder et al., 2016). *In vivo* testing has shown that phages can be used to treat various types of infections in animal models (Hawkins et al., 2010; Dufour et al., 2015; Ghorbani-Nezami et al., 2015; Holguín et al., 2015; Galtier et al., 2016) and also in humans (Kutter et al., 2010; Abedon et al., 2011; Chanishvili, 2012; Rose et al., 2014; Abedon, 2015). Results from these more recent *in vitro* and in *vivo* trials have led to a deeper understanding of the unique nature of phage therapy but have also highlighted the need for further research into their pharmacokinetics and pharmacodynamics (PD/PK).

Often compared to conventional antibiotics in the lay press, the capability to kill bacteria is the only similarity that whole phages and antibiotics share. Therefore, whole phage therapy is often complicated by additional factors and as such possesses unique pharmacokinetics and pharmacodynamics that remain poorly understood. Amongst these unique characteristics is

the poor diffusion of phages as the result of their immense size when compared to antibiotics ($\sim 10^6$ times larger). This means that whole phages cannot be administered in high concentrations ($>10^{10}$ PFU). In order to provide an equivalent amount of "drug" compared to a 10 day course of penicillin (assuming equal minimum inhibitory concentrations and non-replicating phages) over 100 Kg of phages would be required (Bancroft and Freifelder, 1970; Nilsson, 2014). This lack of diffusion and restricted dosage concentration can be offset by the ability of phages to replicate upon finding their target organism.

As with all antimicrobial agents, the ever present shadow of resistance is particularly relevant to whole phage therapies where bacterial exposure to phages provides a co-selective pressure to develop and evade resistance. In the case of conventional antibiotics, targets are often essential metabolic functions, while phages and phage derived products (e.g., endolysins) primarily target surface structures, among the most rapidly changing features in bacteria. The epoch spanning co-evolutionary arms race between phages and bacteria have also resulted in the development of a number of distinct and constantly evolving anti-phage systems, most famously CRISPR-Cas systems (Horvath and Barrangou, 2010), to protect bacteria from infection by phages. In addition to CRISPR-Cas systems, other bacterial resistance mechanisms exist including phage exclusion and restriction modification systems, and have been discussed extensively elsewhere (Hyman and Abedon, 2010). While such systems present a threat to the overall efficacy of a whole phage therapeutic, they are not universally distributed in bacterial species (Grissa et al., 2007; Burstein et al., 2016) and phages also develop counter measures to these resistance mechanisms (Maxwell, 2016).

Although humans are routinely exposed to phages on a daily basis, concern persists over their immunogenicity and overall safety, presenting an additional stumbling block for the adoption of phage therapy. High doses of phage proteins can elicit unwanted side effects from stimulation of the immune system (Gorski et al., 2012; Dabrowska et al., 2014). Due to their classification as biological therapeutics (Rose et al., 2014), both whole phage therapies and therapies based on phage derived products will need to be manufactured under current good manufacturing practices (cGMP) and also adhere to current pharmacopeia requirements that are based on the type of application. This will require not only large scale manufacturing in inert suspension media, something being addressed by small biopharma, but also production of ultrapure preparations conforming to strict endotoxin requirements (<0.5 EU/mL for subcutaneous injections).

CURRENT CLINICAL TRIALS REGULATION

The regulatory foundation for clinical studies and clinical trials in humans is to ethically establish the potential toxicity, efficacy and side effects of new drugs and to prioritize the health of the participants over the generation of results. It is equally important that sufficient data support the claim of potential benefits and that these benefits outweigh anticipated risks. Clinical studies and trials should be carried out in a scientifically correct and transparent manner, be designed to result in trustworthy data and assess the pharmacological properties of the new drug in a stepwise process adapted to available information.

PRE-CLINICAL TESTING

In the current paper, a number of points will be discussed that specifically impact upon individual licensing pathways. In addition, there are a number of pre-clinical testing issues which need to be addressed prior to use in patients regardless of the approval process. These include the need to develop standard testing protocols such as those found in antibiotic (e.g., BSAC or EUCAST; Brown et al., 2016) or microbicide biocide testing (e.g., ASTM E2197) to ensure consistency in results. There is an ongoing shift from classical qualitative assays such as the spot test (host range is assessed by plaque formation) to more quantitative methods such as the efficacy of plating (number of plaques on a target strain compared to number of plaques on the routine host; Khan Mirzaei and Nilsson, 2015), protocols still vary between laboratories. The creation of international standards would ensure the reliability and reproducibility of data.

Standard "efficacy" criteria are utilized by companies seeking to claim activity for chemical microbicides against a particular pathogen (e.g., a defined strain of *S. aureus* as an analog for MRSA). These activities are often under defined environmental and test conditions utilizing a reference strain as the target (e.g., ASTM E2197)[1]. Currently no such standardized criteria exist for whole phages. Although whole phages are unlikely to achieve such large reductions in short time periods (usually $\geq 5\log_{10}$ reduction in <5 min), suitable criteria could be established. These criteria could be based upon a defined lower kill level, the persistence of antibacterial activity over prolonged periods of time, or other virulence characteristics (Borysowski et al., 2014). Such criteria would enable multiple libraries based on lytic activity to be assembled for custom made therapies.

CLASSICAL CLINICAL TRIALS

Clinical trials in the United States must be carried out in accordance with laws in the United States Code, title 21, chapter 9; the Federal Food, Drug and Cosmetic Act and in particular part A of subchapter V: Drugs and Devices (http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/FDCActChapterVDrugsandDevices/default.htm#Part_A; accessed 13th June 2016) under the jurisdiction of the FDA and have influenced the regulations of many other countries due to their comprehensive nature. Within the EU, clinical trials are currently performed in accordance to the Clinical Trials Directive (EU-CTD). This should be superseded in 2016 by the simplified and updated Clinical Trials Regulation

[1]ASTM E2197-11, Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Chemical Germicides, ASTM International, West Conshohocken, PA, (2011), www.astm.org.

(EU-CTR; http://ec.europa.eu/health/human-use/clinical-trials/directive/index_en.htm; accessed 13th June 2016), allowing a single application in one member state to apply to all EU member states which would participate in the trial.

Clinical trials are often broken into four distinct phases (**Figure 2**) following successful pre-clinical studies. These phases increase in complexity and size as a product moves closer to approval. Should approval be granted, all products are then subjected to rigorous routine review as they are used. These stages have been reviewed in detail elsewhere (Pocock, 1983) and are summarized in **Table 2**.

It is estimated that ~US$2.6 billion is required to successfully go from concept to an approved drug (Mullard, 2014). Although some of the initial screening has been taken up by academia (mainly pre-clinical and development work), a significant investment of time and resources is still required from pharmaceutical companies. When compared to drugs developed to treat chronic conditions (e.g., statins), the development of antibacterial agents is economically unviable, due to their comparatively short usage time.

In response to the pressing need to develop new, safe and effective antibacterial agents, additional legislation has been introduced which attempts to modify the approval process, including limited population approval and the incentivization of antibacterial drug development (Brown, 2013; Bax and Green, 2015).

THE APPLICATION OF WHOLE PHAGE AND PHAGE DERIVED PRODUCTS TO "CLASSICAL" CLINICAL TRIAL SCENARIOS

Both whole phages and their derived products will be subjected to the same rigorous clinical trials process as antibiotics. They are also classified as "Therapeutic biological products" and thus subject to the Food, Drug, and Cosmetic Act (http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/FDCActChapterVDrugsandDevices/default.htm#Part_A; accessed 13th June 2016) and also the Public Health Service Act (http://www.fda.gov/RegulatoryInformation/Legislation/ucm148717.htm; accessed 13th June 2016) and under EU Directive 2001/83/EC (Rose et al., 2014; Pelfrene et al., 2016) and would require additional controls over the manufacturing process. Should any changes be made to the manufacturing process, extensive comparability testing would be required to confirm the consistency of the product (Chirino and Mire-Sluis, 2004). Regulators, such as the EMA, are aware of the additional issues faced by phage therapeutics and believe that dialogue with developers will contribute toward a solution (Kingwell, 2015).

For ease of reference, a number of assumptions have been made in the current article as follows:

- A single strictly virulent phage or phage derived product (e.g., endolysin) is selected from pre-clinical studies which produces a suitable level of bacterial reduction for the intended application.

TABLE 2 | Summary of clinical trials testing requirements.

Phase	Aim	Cohort size	Notes
Phase I	Determination of safety	20–100	• 1st in human studies using healthy volunteers • Evaluation of dosing while monitored • Determination of adverse effects
Phase II	Determination of efficacy	100–500	• Determination of efficacy in target population • Evaluation of side effects
Phase III	Confirmation of efficacy	1000–5000	• Verification of efficacy in target population • Evaluate rarer side effects • Comparison to gold standard treatment
Phase IV	Safety surveillance	–[a]	• Monitoring of routine use to ensure no adverse side effects

Adapted from (Pocock, 1983). [a]*Not applicable.*

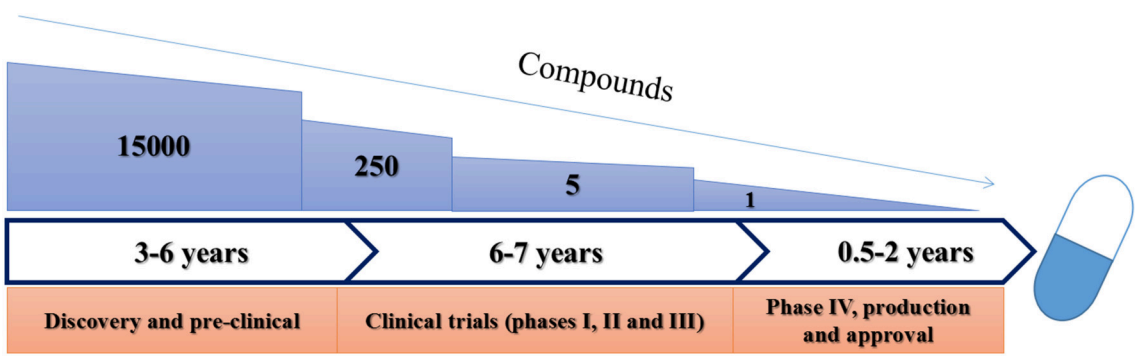

FIGURE 2 | Schematic representation of current FDA approval procedures for anti-infective drugs.

- The phage or phage derived product has been subjected to appropriate pre-clinical *in vivo* testing to determine the toxicity, immunogenicity, and dosing of the treatment.
- All products should be produced according to cGMP, as determined by local regulators, as well as being soluble in, and compatible with, commonly used physiological solutions (e.g., saline) or other physiologically inactive media.
- All components of a phage or phage product cocktail must have been shown to be acceptable as stated above for single phages. In addition, the dynamics between the individual components of the cocktail should also be assessed prior to use.

Initial clinical testing (Phase I) will not vary greatly between antibiotics, whole phages or their derived products and as such should be relatively simple to perform with the appropriate approvals. The routine exposure of humans to phages provides the immune system with a low level of circulating phage-specific antibodies (Kucharewica-Krukowska and Slopek, 1987) and subsequent exposure as part of a therapy may compound this. Indeed a number of *in vitro* and *in vivo* studies have shown that phages stimulate the innate and adaptive immune systems in a phage specific manner (Dabrowska et al., 2014; Majewska et al., 2015) and potentially in a protein specific manner (Dabrowska et al., 2014). However, a number of studies performed with cocktails of whole phages have suggested that phages are harmless when ingested (**Table 3**; Bruttin and Brussow, 2005; Sarker et al., 2012) or applied topically (Rhoads et al., 2009). This lack of response may be in part due to the degradation of phages as they transit the digestive system, reducing the number that come into contact with immunostimulatory cells (Abedon, 2015), but also due to varying degrees of sensitivity between different cell types.

Despite this initial suggestion of safety under Phase I conditions, potential safety issues will remain during Phases II–IV as "rarer" side effects are sought. At these stages, drugs under evaluation are subjected to trials of efficacy in a population who suffer from the particular disease under investigation. Due to the nature of the lytic phage lifecycle, it is not inconceivable that the active replication of phages at a site of infection could produce side effects, such as toxic shock, as bacterial debris is released. Such issues could potentially be anticipated and avoided by the selection of phages which exhibit different properties such as lower virulence or through the combination of phages with conventional antibiotics. Although this may suggest that whole replicating phage therapies could be consigned to topical application further research is required, particularly if the issue is addressed through the incorporation of anti-endotoxin adjuvants (de Tejada et al., 2015; Valera et al., 2015).

During Phase II and III studies additional complications arise when trying to recruit a statistically relevant homogenous population to study (Rose et al., 2014). In the case of diseases caused by a single bacterial species (e.g., cholera), this may be due to a low incidence in the general population or, and more likely, the disease can be caused by multiple organisms (e.g., diabetic foot ulcers). It is therefore likely that clinical trials on phages will be based on long term or multi-site studies in order to obtain representative population sizes and could be facilitated by the introduction of the EU-CTR. The introduction of the EU-CTR could also enable trials to be coordinated from specialist phage therapy centers allowing for the distribution of specialist knowledge and products.

Although the combination of multiple phages into a cocktail compensates for a limited host range (Bruttin and Brussow, 2005) a number of compromises are made. The increased complexity of multi-phage cocktails will dilute the concentration of the individual phage components due to their size, and also introduce competition of phages for binding sites, both of which could compromise the treatment (Nilsson, 2014).

Pre-made phage cocktails can be designed to target either against uncharacterized (multiple phages targeting multiple bacterial species), or typed bacterial infections (multiple phages targeting a single bacterial species). Additionally, patient specific cocktails can be produced in which phages are selected from a pre-existing library against the patient's specific strain (Pirnay et al., 2011). While the manufacture of pre-made cocktails would be tightly controlled and mass produced under cGMP in order to satisfy regulatory requirements (Parracho et al., 2012; Rose et al., 2014) it could decrease the production cost per dose. Pre-made cocktails would also require supplementary approval as cocktail components are modified to compensate for the development of bacterial resistance. The clinical usefulness of pre-made cocktails would be limited due to the shifting nature of epidemic strains; however, the rate at which resistance develops under therapeutic conditions is currently unknown. In theory at least, a pre-defined cocktail should be able to successfully navigate the current regulatory process assuming appropriate non-inferiority (drugs under investigation possess similar levels of activity compared to standard treatment) trial designs. However, it remains unclear if additional approval would be required as components of the cocktail change.

Although patient specific cocktails may provide better overall results (due to the tailored nature of the treatment) these would present additional challenges in order to gain regulatory approval. Classical trials of patient specific cocktails would have to be designed to target specified bacterial strains within the same species, further reducing the available population which could be recruited and require multi centered trials to be undertaken. In theory at least individual approvals would be required due to their unique composition. In order to compensate for the variety of potentially infectious strains, patient specific cocktails would require libraries of pre-approved phages to be developed. This would allow cocktails to be assembled on a case by case basis, currently an unprecedented move, although the stockpiling of vaccines and some antitoxins could be considered to be a suitable analog (Bodas et al., 2012; Martin et al., 2012).

As previously mentioned, the overall cost for the complete (pre-clinical to Phase III) development of a novel drug is astronomical (estimated to be US$2.6 billion; Mullard, 2014) and represents a significant obstacle for the broad evaluation of phage therapy in human populations. This cost would be on a per cocktail basis (assuming cocktails were pre-defined and manufactured) and probably equate to those encountered by an antibiotic or phage derived protein. When new strains arise, for which the pre-defined cocktail is not approved, treatment could

TABLE 3 | Current clinical trials for phage based therapy in humans.

Trial number	Study title	Status (date of completion)	Summary of trial	Published research articles
NCT02664740	Standard treatment associated with phage therapy vs. placebo for diabetic foot ulcers infected by *S. aureus* (PhagoPied)	Not yet recruiting	• Multicenter trial comparing phage impregnated dressing (10^7 PFU/mL) to a placebo dressing • Dressings to be replaced at Day 7 and 14 • Wants to recruit 60 participants • Measuring wound healing over 12 weeks • Presence/absence of bacteria and antibiotic resistance	–[a]
NCT02116010	Evaluation of phage therapy for the treatment of *Escherichia Coli* and *Pseudomonas aeruginosa* wound infections in burned patients (PHAGOBURN)	Recruiting (July 2016)	• Phase I/II multicenter trial comparing phage cocktails against Silver Sulfadiazine • Time taken to get a persistent reduction of bacteria relative to bacterial content at D0 • Assessing tolerance to the treatment • Assessing level of clinical improvement	–[a]
NCT01818206	Bacteriophage effects on *Pseudomonas aeruginosa* (MUCOPHAGES)	Completed (April 2012)	• Induced sputum samples taken from 59 CF patients • *Ps. aeruginosa* count after 6 and 24 h exposure to phage cocktail • Phage counts after 6 h	Saussereau et al., 2014
NCT00945087	Experimental phage therapy of bacterial infections	Unknown (Last Updated Sept 2013)	–[a]	
NCT00663091	A prospective, randomized, double-blind controlled study of WPP-201 for the safety and efficacy of treatment of venous leg ulcers	Completed (May 2008)	• Phase I safety study evaluating an 8 phage cocktail (each phage component approx. 10^9 PFU/mL) • Desired enrollment of 64	Rhoads et al., 2009
NCT00937274	Antibacterial treatment against diarrhea in oral rehydration solution	Terminated (Jan 2013)	• Comparison of 2 separate T4 phage cocktails against standard oral rehydration solutions in ETEC and EPEC infections • Desired enrolment of 120 • Assessment includes safety tolerance and reduction of stool volume and frequency	Sarker et al., 2016

Data obtained from http://www.Clinicaltrials.gov (09/03/16). [a]Not applicable.

still be carried out under compassionate usage or "off license." However, in the case of patient specific cocktails, approvals may have to be obtained on a phage by phage basis, prior to combination into cocktails and could potentially, increase overall costs by orders of magnitude.

The biology and unique, but poorly understood, PD/PK of whole phage based therapies, may in actuality reduce their viability as antibiotic replacements for the treatment of bacterial infections in humans. In the case of PD, their large size and poor diffusion through non-aqueous mediums would present challenges if used as a systemic treatment. This would require potentially huge doses of phage to be administered in order to achieve a therapeutic effect. In terms of pharmacokinetics, large phage doses would be cleared efficiently from the body by the immune system and could prevent the establishment of a productive phage infection. The administration of large doses of phages would increase the probability of phages being able to reach the site of infection prior to being removed by the immune system. In addition to this, the overall immunostimulatory capacity of phage could be reduced through complex formulation by masking the phages (Kim et al., 2008) or through modification of phages to alter immunostimulatory proteins (Dabrowska et al., 2014).

Although many of the issues raised here have been presented as phase specific, they in fact transcend the individual trial phases and represent incompatibilities within the current approvals process itself. Indeed, for patient specific cocktails, it is highly improbable that current regulations would allow for the approval of a library rather than requiring the approval of each individual phage, drastically increasing the overall cost.

Conversely, phage derived products (e.g., endolysins) may address some of these limitations and have attracted some attention from commercial entities (**Table 2**). Despite being defined as "therapeutic biological products" their activity kinetics, and probably approval pathways, would be more akin to antibiotics than whole phage cocktails. The ability to produce them as recombinant proteins in a non-target vector means that overall manufacturing and purification processes could

be adapted from currently existing methods (such as those used to produce insulin). This would allow for classification at a substantially higher level of detail than is possible with whole phage cocktails. The activity of endolysins has also been shown to transcend single bacterial strains in Gram positive pathogens such as *S. aureus* (Fischetti, 2016). This would not only enable trials to be performed on larger populations but could also increase their attractiveness to larger scale pharma companies, as they could be used to target multiple conditions caused by a particular bacterial species. However, they would be of limited effectiveness against polymicrobial infections. Many of the derived endolysins currently described in the literature target Gram positive pathogens (Fischetti, 2010; Nakonieczna et al., 2015). Further research is needed to evaluate their efficacy against Gram negative pathogens due to differences in cell wall composition as well as more research on Gram negative specific endolysins (Dong et al., 2015; Oliveira et al., 2016). Additionally, other phage derived proteins such as holins and tailspikes may provide suitable alternatives to endolysins for Gram negative pathogens (Saier and Reddy, 2015).

ADAPTIVE LICENSING FRAMEWORKS

The process for gaining regulatory approval for novel antibiotics is a long and time consuming process, which is further complicated when whole phages are applied to these proceedings. Although the process itself is optimized for certain types of drugs, regulators believe that the current trials legislation is "adequate" for use with bacteriophage based therapies (Verbeken et al., 2012; Pelfrene et al., 2016). However, many researchers engaged in the field actively disagree with this, as the current approvals procedures are too rigid and too costly in terms of time and money. They have more recently suggested that current pathways need to be modified or novel pathways need to be developed for use with phages (Verbeken et al., 2014; Kutter et al., 2015; Young and Gill, 2015).

Multiple initiatives have been taken by both the FDA and Europe Medicines Agency (EMA) to simplify and shorten the approvals process for drugs while maintaining standards. They are not designed for antibacterial drugs or, more specifically, phage based therapeutics. These frameworks include an EMA pilot project on adaptive licensing initiated in 2014 (AL or adaptive pathways) and encompasses six undisclosed products. The pilot study, which is due to report later in 2016, seeks to investigate how current regulations can be optimized for the approval of new drugs in cases where there is a high medical need. The pilot also seeks to determine which criteria should apply for drugs that can be approved in a graduated simplified process.

Introduced to the US Senate in January 2015, the Promise for Antibiotics and Therapeutics for Health (PATH) Act (S.185; https://www.congress.gov/bill/114th-congress/senate-bill/185/text; accessed 13th June 2016) is an amendment to section 506 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 356). This amendment proposes the creation of a "limited population pathway for antibacterial drugs" that will allow for the approval of antibacterial drugs within a highly defined population without the need for clinical trials through the development of a Benefit-Risk profile that reflects the "severity, rarity, or prevalence" of the infection. Although the decision making process could inevitably be informed by both traditional (e.g., survival), alternative (e.g., bacterial clearance), and small clinical data sets, the process may also take into consideration other supplementary information such as non-clinical susceptibility and pharmacokinetic data. However, the supplementary set of considerations will be decided on a drug by drug basis. Once approved, product labeling will reflect the limited population that the drug can be used on and subjected to post approval monitoring. Subsequent approval for use within a wider population can be sought, but it is not clear if this would require full clinical trials or if off license use would be permitted.

AL pathways are iterative processes in which treatment outcomes are used to inform the ongoing trial, through the involvement of all stakeholders, as well as input from independent scientific advice. The iterative process can, as in the EMA pilot project, be based on different conditions including: (i) the introduction of more steps, starting with a small highly defined patient population which is expanded as more information becomes available (ii) conditional approval for a product that is granted based on existing data, or (iii) adopting a centralized compassionate use of a new drug.

ADAPTIVE LICENSING FOR PHAGES

When compared to classical trials, AL pathways may provide additional flexibility that would enable whole phage therapeutics and their derived products to be approved for clinical use. These opportunities would come in the form of initial limited population testing and potentially through the use of non-traditional surrogate endpoints.

Regardless of the pathway employed, phage clinical trials will inevitably consist of a series of compromises due to the complex interplay between infection type, causative agent and therapeutic strategy. Trial outcomes will also shift depending on additional priorities such as clinical need, scope of the trial, anticipated efficacy of treatment, and overall cost. In addition to this, trials could be based around a multitude of different formulations, each possessing advantages and disadvantages (**Figure 3**).

FORM OF THERAPY

As previously mentioned, phage therapy may involve treatment with single phages, phage derived products e.g., endolysins, or cocktails. Host range considerations would limit the available population that could be treated with single phage preparations, whereas the formation of phage cocktails would be able to increase the number of patients that could be treated. The number of potential recruits for trials could also be increased for disease states which cause seasonal outbreaks of a single clonal type in a confined area (e.g., hospital acquired *Clostridium difficilie* infections; Furuya-Kanamori et al., 2015). Trial participants could be easily recruited for clinical trials of a single phage, particularly if the target organism is a commonly

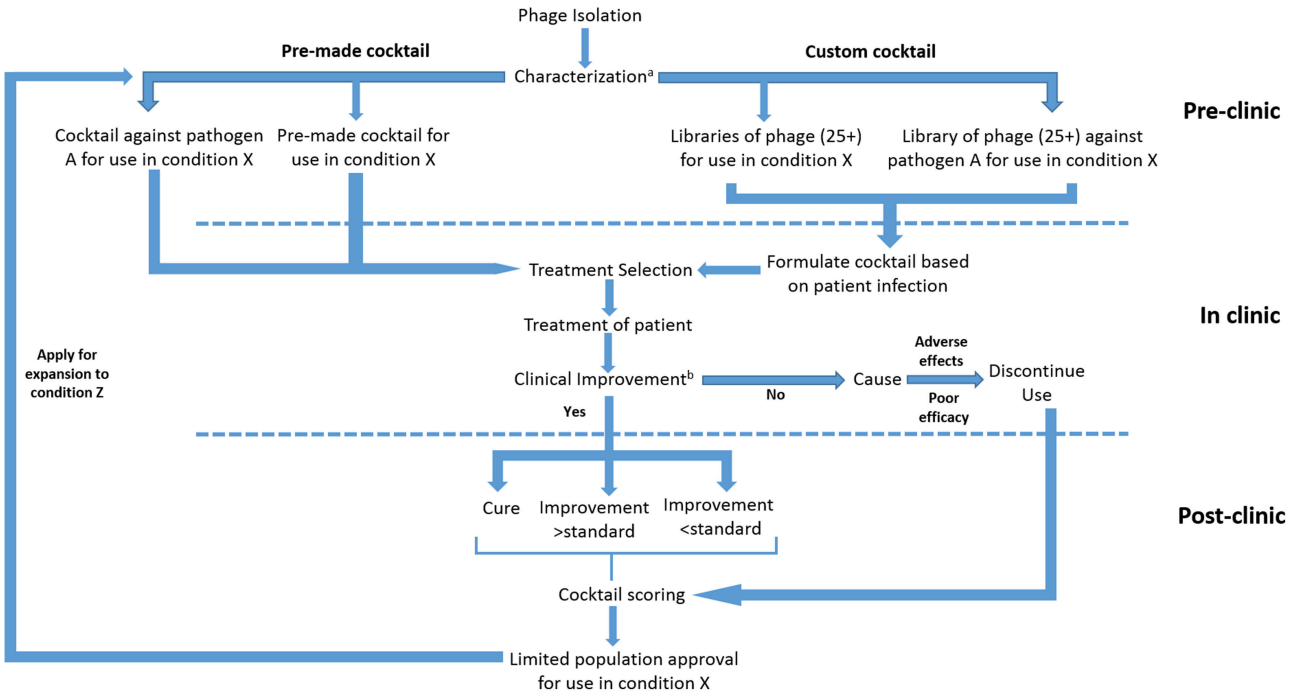

FIGURE 3 | Proposed submission of whole phage based products under an adaptive licensing framework. [a]Characterization to be based on genotypic analysis and lytic activity. [b]Improvement to be characterized on the basis of classical and surrogate endpoints.

occurring pathogen (e.g., MRSA). The recruitment process would be also be facilitated by a centralized approvals process (e.g., EU-CTR) which would enable multi-centered trials to be performed with a single application. However, many infecting bacterial strains are temporally or spatially restricted, limiting the availability of participants.

Phage cocktails, on the other hand, can increase the number of strains susceptible to infection by one or more of the components which make it possible to target common bacterial infections caused by different strains in different patients and thus facilitate the recruitment of participants. Cocktails can be pre-made and targeted against common pathogenic strains or custom made, either upon the emergence of a particular strain or to fit the requirements of individual patients (Pirnay et al., 2011).

Pre-made Cocktails

Highly defined pre-made cocktails should be able to fit into existing AL frameworks as it would be relatively simple to define a limited population, although this would not be able to compensate for differences between causative strains. Should the causative strain be specified (e.g., *Pseudomonas aeruginosa* PAO1) this would further limit the population which could be recruited, therefore a multi-centered approach would be needed and could be expedited by the modified EU-CTR. To compensate for shifting global trends, clinically relevant bacterial collections should be assembled and distributed which would enable easier isolation of suitable phages. It should also be noted that pre-defined cocktails are less flexible to the rise and fall of new bacterial strains and would ultimately be susceptible to the development of bacterial resistance. The overall efficacy would decrease over time in which a new cocktail would need to be developed and approved for use.

Custom Cocktails

Custom made cocktails are one way to address the development of bacterial resistance against phages. In the case of bacteria which harbor phage resistance systems (e.g., CRISPR), phages encoding specific anti-defense mechanisms e.g., anti-CRISPR systems (Bondy-Denomy et al., 2013) could be given higher priority even if their infection characteristics are not as good as other phages. Several phages targeting different surface receptors could be applied simultaneously or serially, resulting in a synergistic effect and could reduce the potential for resistance developing (Schmerer et al., 2014) although other criteria could be used for the selection of cocktail components (Chan and Abedon, 2012). The inducement of synergy between phages would also be a good strategy for long term treatment of deep infections. In such infections, phages would have difficulty reaching their targets and would be cleared by the immune system reducing their overall number. By inducing synergy a smaller overall number of phages would be required to reach the site of infection as each phage component would be able to establish a productive infection. However, as with all phage trials there are arguments that the overall number of participants in custom cocktail trials would be limited to just one (Eichler et al., 2015) as infecting strains, and therefore cocktail composition, would vary on a patient to patient basis. As such it may be advantageous to inform AL trials based on a pre-characterized

library of phages against a defined pathogen in a defined condition (e.g., *Ps. aeruginosa* burn infections), although this would require a significant redesign of approvals processes.

Creation of a Pre-characterized Library of Phages and Selection for Use in Patients

A long-term possibility for the implementation of whole phage therapeutics would be to create phage libraries containing the most efficient phages against the most severe pathogens, e.g., multiresistant Gram-negative bacteria. This could be done by initially pooling existing phage banks (most of them maintained by research institutions) into common libraries, followed by the continuous isolation and addition of new phages. Copies of the library would be stored in national phage depositories or major hospitals. This would shorten the time for finding highly efficient matching phages and assembling cocktails as well as facilitate an approval process. Individual phages would initially be characterized *in vitro* (as with most isolation and characterization papers) using structural, genomic (i.e., absence of lysogenic properties), host range, and efficacy analyses (Malki et al., 2015; Sauder et al., 2016). However, and more importantly, the pre-characterization could also establish safety and efficacy including suitable *in vivo* testing in animal models before phages are added to the library. It is important to ensure that even phages which do not meet the required *in vitro* efficacy criteria (e.g., insufficient lytic activity) are not discarded as they may perform better *in vivo* (e.g., less immunostimulatory), or possess greater efficacy against future epidemic strains.

The assembly of pre-approved phage libraries could potentially prove advantageous as it would allow for the creation of multiple cocktails to target an individual infection type within a single library thereby increasing the population size that could be recruited. Only the cocktails themselves would need to be tested for safety and efficacy since individual phages in the library would already be fully tested. This approach would also allow for the creation of additional libraries for phages which appear to be less active or possess a restricted host range. Indeed the possibility of creating multiple or tiered libraries (as is the case with multiple lines of antibiotics) would allow additional flexibility as resistance to individual phages develops. However, as the complexity of cocktails increases to treat polymicrobial infections, or as multiple tiered libraries are assembled, the overall cost and time required to complete trials would increase.

It should be noted that the concept of pre-approved libraries would require a radical shift in the thought processes of regulatory agencies and would require the development of new assessment criteria. These criteria could include the absence of non-toxicity regardless of the combination of phages used, in combination with a "minimal" activity level of each phage. Such a shift in thought process would also ultimately lead to the development and approval of libraries of phage derived antibacterial proteins. By approving both phages and their derived proteins as libraries rather than on an individual basis, the overall number of trials would be reduced due to increased flexibility of the drug and potentially an increased treatment success rate.

Patient Criteria and Emergency Procedures

Following the successful formation of a pre-characterized library or specified cocktail, patients would be recruited on the basis of confirmed infection type (i.e., a specified bacterial agent in a specified disease state). As in the EMA pilot studies, the trial would progress iteratively, starting with small groups of participants and resolving uncertainties before expanding the trial into new populations. Adverse effects influenced by rare human genetic traits may be problematic for this type of AL approach, but further research is required. As a consequence, the initial (human) testing of phages or cocktails from the libraries should be conducted in non-life threatening topical infections (e.g., diabetic foot infections). Any target infection should also have at least one additional treatment available as a safety precaution. Should the application of the cocktail result in Serious Adverse Events/Serious Adverse reactions, Suspected Unexpected Serious Adverse Reactions or no measurable clinical benefit, the decision to remove a patient from the treatment should be made quickly and alternative treatment applied as soon as possible.

Assessing Outcomes and Expansion of Populations

The necessary expansion from small groups of trial participants to larger cohorts during adaptive pathway trials would be contingent on the outcomes that can be assessed given the actual cohort size. Classical clinical trials often use well-defined and clinically relevant endpoints like patient survival time, resolution of infection, decrease in lesion size or perceived symptoms, but surrogate endpoints based on biomarkers for indirect assessment can also be applied (Fleming and Powers, 2012).

In the initial stages, classical endpoints should be included in adaptive pathway phage therapy trials, but pharmacological assessment will be as important. Surrogate endpoints will probably be of greater importance for phage therapy trials. Increase in phage titers, reduction of bacteria load or infection parameters (e.g., CRP) and absence of additional pro-inflammatory responses may indicate that the treatment has a positive effect and that the study can be widened. Therefore, phage trials under AL would be designed as non-inferiority trials, in which the intervention is compared to the conventional therapy (e.g., antibiotics) to establish a similar level of overall effect (D'Agostino et al., 2003) rather than having to demonstrate superiority.

If applied in a pre-formed library format for patient specific cocktails, individual phage components could potentially be scored to further inform and develop the library. Such a scoring mechanism would not only require the treatment outcome to be established, but the actual role of the individual phage in that outcome and would require the ability to differentiate between the components of the cocktail.

AL pathways offer many possibilities for the approval of whole phages. However, each of these different avenues require compromises which will subsequently impact the efficacy of the treatment and on the overall cost and time required to complete

trials (**Table 4**). In the case of cocktails the time and cost to reach the clinic will increase significantly if it is necessary to approve each individual component of the cocktail separately. However, if radical action is taken and libraries of phages against specified pathogens are approved, this could potentially counter the increased cost and time.

Although it is likely that phage derived proteins will not suffer adversely when trying to gain regulatory approval, AL pathways could still prove beneficial. The use of a limited population approach would enable data to be obtained while informing future clinical studies of different disease states, particularly if developers are interested in systemic application. In addition to this, classical and surrogate endpoints could be utilized that could be derived from antibiotic trials (Cornely et al., 2012; Verduri et al., 2015).

"RIGHT TO TRY" LEGISLATION AND "COMPASSIONATE USE"

The Code of Federal Regulations (CFR) Title 21, Chapter I, Subchapter D, Part 312, Subpart I (http://www.ecfr.gov/cgi-bin/text-idx?SID=43f054659224216924a6379ef9602c2b&mc=true&tpl=/ecfrbrowse/Title21/21tab_02.tpl; accessed 13th June 2016) and European Regulation 726/2004/EC (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:136:0001:0033:en:PDF; accessed 13th June 2016) govern the expanded access of non-approved medications to patients that have been submitted for approval by regulatory agencies. Although this is often under compassionate use guidelines where no alternative treatment exists (Whitfield et al., 2010) there are increasing calls to amend the laws in the US to make this process easier through additional legislation such as the H.R 3012 (Right to Try Act; https://www.congress.gov/bill/114th-congress/house-bill/3012; accessed 13th June 2016). Introduced to Congress during July 2015, H.R. 3012 would allow Phase I experimental drugs, biological products, or devices to be used in terminally ill patients.

Although such regulations would allow for the use of phages on individual patients in the US under Part 312.310, expansion to an intermediate patient population size (Part 312.315) would be possible, but potentially difficult. While there have been a limited number of cases in which phage cocktails have been approved for compassionate use this process is not routine (Rhoads et al., 2009; Khawaldeh et al., 2011). However, should resistance rates continue to increase, and the number of available drugs decrease further, phage therapy may be the only remaining therapeutic option.

In addition to cGMP and other specific requirements, informed consent from patients is required for compassionate use. While this should be relatively simple, the lack of public awareness surrounding phages may be detrimental to recruitment. This lack of public awareness could be circumvented by the formation of trials in EU member states possessing specialized phage centers (such as the Institute of Immunology and Experimental therapy in Wroclaw Poland) as part of an EU-CTR application in combination with compassionate usage in other parts of Europe which has been suggested by researchers in the field (Kutter et al., 2015).

UTILIZATION OF ADDITIONAL DATA SOURCES

The clinical use of phages in specialized centers in Eastern Europe has generated immense amounts of data, little of which is published in Western scientific literature (Miedzybrodzki et al., 2012). As some of these centers now lie within the EU, data generated post membership should conform to Western standards and could potentially form the basis of a meta-analysis or systematic review of clinical phage therapy when combined with more recent trial data that has been generated.

In order to utilize such data that has been generated prior to EU membership, or for countries whose regulatory frameworks may differ to EU and FDA standards, criteria would need to be established in order to assess the overall quality of the work performed. This could be done on the basis of the achievement of an appropriate clinical outcome (i.e., duration of hospitalization or resolution of infection) or an assessment of the methodology and trial design prior to incorporation into meta analyses.

CONCLUDING REMARKS

Despite the pressing need to develop new antibacterial agents, the approval rate of anti-bacterial drugs remains low when compared to other forms of drug due in part to both economic and scientific issues. Phage derived products, such as endolysins, are likely to be suitable for classical clinical trials procedures due to their similarities with conventional antibiotics. However, in the case of whole phage therapies, currently available mechanisms are not suitable, requiring large patient cohorts and extensive resources. In this article the limitations of current clinical approval pathways, as well as possible alternative pathways for the approval of phage therapy, have been discussed and summarized (**Table 5**).

While the current article is by no means exhaustive on every potential pathway that could be employed for the approval of phage based therapies for human use, it hopefully sparks discussion and debate on the nature of clinical trials and the need for more flexible regulations when dealing with phages and their derived products. For phages that are genetically similar (>95%) this could include additional accelerated or automatic approval pathways. This would be particularly useful for those phages whose major components (e.g., capsids) are identical but whose host range and efficacy is influenced by small changes in tail fiber composition (Ando et al., 2015; Goren et al., 2015). However, genetic engineering of whole phages (Ando et al., 2015), or the creation of wholly artificial phages from sequence data (Smith et al., 2003), would require approvals pathways to be revisited in the future as these technologies approach clinical readiness. In the case of customizable cocktails taken from pre-licensed libraries, suitable regulatory criteria need to be developed. This would in essence separate phage approvals from the normal

TABLE 4 | The implementation of adaptive licensing pathways for single phage and pre-made or custom phage cocktails.

	Single phage	Pre-made cocktails		Custom cocktails		
		Untyped infections	**Typed infections**	**Single species typed infections**	**Multi-strain typed infections**	**Polymicrobial typed infections**
Phage selection	• One phage with high efficacy against a typed bacterial strain.	• Multiple phages targeting an untyped infection.	• Multiple phages, compromising between efficacy and host range.	• Multiple phages targeting a single typed bacterial strain.	• Multiple phages targeting multiple typed bacterial strains.	• Multiple phages targeting multiple typed bacterial species.
Aim	• Treat MDR/XDR strains only.	• Treat bacterial infections based on symptoms.	• Treat infections caused by multiple strains of the same species.	• Treat patient specific infections caused by a single typed species.	• Treat multiple strains of the same species in a single patient.	• Treat typed polymicrobial infections in a single patient.
Advantages	• Easy to change phages. • Easy analysis.	• Easy recruitment to trials.	• Easy recruitment to trials. • Only limited phage library needed.	• Predicted high efficacy. • Avoids emerging bacterial resistance.	• Wide application.	• Wide application.
Disadvantages	• New phages require additional trials. • Unpredictable results.	• Poor patient recovery rate. • Unpredictable results. • New formulation means new trial.	• Cocktail obsolescence. • New formulation means new trial.	• Unique cocktails increase recruitment difficulty to trials. • Cocktail obsolescence. • Large phage library needed.	• Every cocktail unique, more difficult to recruit trial participants. • Necessary to reformulate cocktail as new strains appear. • Large phage library needed.	• Every cocktail unique, more difficult to recruit trial participants. • Necessary to reformulate cocktail as new strains appear. • Multiple large phage libraries needed.
Iteration	• Phage is discarded or formulation changed as new data emerges.	• Non-effective or hazardous phages are removed as data emerges. • Additional phase I testing if there is poor efficacy or immunogenicity.	• Non-effective or hazardous phages are removed as data emerges. • Additional phase I testing if there is poor efficacy or immunogenicity.	• Cocktail composition altered as data on phages emerge. • Additional phase I testing if there is poor efficacy or immunogenicity.	• Cocktail composition altered as data on phages emerge. • Additional phase I testing if there is poor efficacy or immunogenicity.	• Cocktail composition altered as data on phages emerge. • Additional phase I testing if there is poor efficacy or immunogenicity.
Design	Single site long term or multi-site short term	Single site long term or multi-site short term	Single site long term or multi-site short term	Multi-site long term	Multi-site long term	Multi-site long term
Implementation time	+	+	+	++ or +++*	++ or +++	+++
Cost	$	$$	$$	$$$	$$$	$$$

**Time to implementation would be affected by the form of treatment chosen. Pre-approved libraries could be implemented faster should suitable criteria be developed.*

TABLE 5 | Summary of current and possible alternative pathways as applied to whole phage and phage derived therapeutics.

	"Classical" licensing	Adaptive licensing	Compassionate use
Advantages	• "Gold" Standard • Already established for antibacterial drugs • Would be suitable for phage derived products • Additional legislation being introduced to streamline procedures for antibacterial drugs	• Limited population approvals • Iterative process which can inform future work • Can be adapted for pre and custom phage cocktails	• Immediate clinical usage • Data could be used to inform future work • Could be utilized for all forms of phage therapy
Disadvantages	• Recruitment for trials • Cost • Reformulation would require additional trials	• Varying degrees of complexity • Limited population approvals	• Limited to a single patient basis • Not actually approved for use
Other considerations	• Likely that only highly defined products would be able to succeed, limiting success	• Approval of predefined libraries would require wholly new approvals process	• Lack of public awareness of phages
Time to implement[a]	++	++ or +++*	+
Cost to implement[a]	$$$	$$ or $$$*	$

[a]*Assumption has been made that "Classical" licensing is a baseline. *Both cost and time to implementation would be affected by the form of treatment chosen. Pre-approved libraries taking longer and costing more to achieve.*

biological therapeutics approvals and would require a substantial shift in the collective mindset of regulatory agencies.

Perhaps the most radical possibility would be to establish a centralized phage bank under governmental control from which phages could be isolated, collated, tested, and distributed on a case by case basis to be used in compassionate use treatments or AL trials. Data and treatment outcomes could then be collated by the phage bank to provide greater insight into phage therapy as a whole. This would not only remove the commercial element to development, but would provide direct to clinic access for therapies and also enable a greater degree of control to be exerted over treatment potentially reducing phage resistance rates. Such a system could be developed within existing public health organizations (e.g., Public Health England, UK or Folkhälsomyndigheten, Sweden) as these organizations are responsible for collating data on antibiotic resistance trends and provide reference laboratory facilities, but would require a substantial initial investment to establish and thus may be unpalatable in the current economic climate.

While no phage specific approvals pathway currently exists, such a pathway could be developed with suitable engagement between regulators and researchers. This pathway could be based on existing guidelines, where products which have been successfully completed phase I clinical studies are applied on a case by case basis under compassionate use guidelines. However, in order to gain widespread adoption it may be better to base phage approvals on AL principles, whereby approval is granted for small specified populations which can then be expanded upon as post-approval data is gathered.

Regardless of the pathway implemented, the overall cost of drug development and the poor return on investment of antibacterial agents will remain one of the defining development issues. Due to the abundance of phages in the environment, patents may be circumvented relatively easily as new phages are isolated and would therefore reduce the potential level of interest from traditional pharmaceutical companies.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Bacteriophage Procurement for Therapeutic Purposes

Beata Weber-Dąbrowska[1,2], Ewa Jończyk-Matysiak[1], Maciej Żaczek[1], Małgorzata Łobocka[3,4], Marzanna Łusiak-Szelachowska[1] and Andrzej Górski[1,2,5]*

[1] Bacteriophage Laboratory, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [2] Phage Therapy Unit, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [3] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland, [4] Autonomous Department of Microbial Biology, Faculty of Agriculture and Biology, Warsaw University of Life Sciences, Warsaw, Poland, [5] Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland

Bacteriophages (phages), discovered 100 years ago, are able to infect and destroy only bacterial cells. In the current crisis of antibiotic efficacy, phage therapy is considered as a supplementary or even alternative therapeutic approach. Evolution of multidrug-resistant and pandrug-resistant bacterial strains poses a real threat, so it is extremely important to have the possibility to isolate new phages for therapeutic purposes. Our phage laboratory and therapy center has extensive experience with phage isolation, characterization, and therapeutic application. In this article we present current progress in bacteriophages isolation and use for therapeutic purposes, our experience in this field and its practical implications for phage therapy. We attempt to summarize the state of the art: properties of phages, the methods for their isolation, criteria of phage selection for therapeutic purposes and limitations of their use. Perspectives for the use of genetically engineered phages to specifically target bacterial virulence-associated genes are also briefly presented.

Keywords: bacteriophage isolation, therapeutic phages, experimental phage therapy, treatment of bacterial infections, antibiotic resistance

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Lei Dai,
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Jean-Paul Pirnay,
Queen Astrid Military Hospital, Belgium

****Correspondence:***
Beata Weber-Dąbrowska
weber@iitd.pan.wroc.pl

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INTRODUCTION

The post-antibiotic era in which minor injuries and common infections can kill – because of lack of drugs or their ineffectiveness – nowadays is not an apocalyptic fantasy, but a real 21st century possible threat (WHO, 2014). For example, ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are extremely resistant to multiple antimicrobial agents (Moellering, 2010) and are a serious challenge in medicine today.

According to the National Institutes of Health (NIH, USA), phages are innovative components that may be used to combat microbial resistance (NIH, 2014). Clinical application of phage preparations is associated with well-described and -characterized processing, and its influence on phage stability during preparation and under storage conditions should be verified and described in detail, especially as it is needed for phase III clinical trials (Vandenheuvel et al., 2015). As suggested, personalized phage therapy is probably more effective than *prêt-à-porter* preparations, as it is based on tailored preparations which can be adjusted to changing bacterial pathogens (Keen, 2012; Mattila et al., 2015).

STATE OF THE ART

Bacteriophages are viruses which have the ability to multiply only in bacterial cells, and they are detectable almost everywhere where live bacteria exist (they have been isolated from all environments and ecological niches, e.g., inland waters, hot springs, salt water, soil, cold water) (Lin et al., 2010; Zheng et al., 2013; Zhan et al., 2015). It is estimated that in the biosphere there are approximately 10^{30-31} phages (Hendrix et al., 1999; Hendrix, 2002), which is 10-fold higher than the total number of bacterial cells (Abedon et al., 2011a). They are an inherent element of the human microbiome, and therefore they are well tolerated when used in phage therapy (Międzybrodzki et al., 2012; Reyes et al., 2012).

A serious medical and social problem results from the increasing antibiotic resistance of bacterial strains (Arias and Murray, 2009; Magiorakos et al., 2012; Deris et al., 2013; WHO, 2014). The pharmaceutical industry is withdrawing from research and development on new antibiotics due to unprofitability of the venture and the risks of development of resistance in bacteria (Clarke, 2003).

Initially, bacteriophages were isolated from stool samples of patients suffering from diarrhea and were first successfully used in the treatment of bacterial dysentery (D'Hérelle, 1917). Hankin was the first to observe that the water of the Ganga and the Yamuna rivers (India) had antiseptic properties; in particular, water samples from them were able to destroy *Vibrio cholerae* (Hankin, 1896; Abedon et al., 2011b).

In humans phages have been successfully used in the treatment of a wide range of infections, both local and systemic (Weber-Dąbrowska et al., 2001, 2003; Borysowski and Górski, 2008; Międzybrodzki et al., 2012; Borysowski et al., 2014; Rose et al., 2014). This is due to the fact that they have many features which give them advantages over antibiotics, e.g., phages multiplying at the site of infection where there are bacteria sensitive to them (Abedon and Thomas-Abedon, 2010; Loc-Carrillo and Abedon, 2011; Kutter et al., 2012). Interestingly, development of the resistance of bacteria to antibiotics does not parallel the development of phage resistance in bacteria, although the acquisition of resistance to phages by bacteria may pose a significant problem (Loc-Carrillo and Abedon, 2011). Therefore, as mentioned, phages can be used to treat infections caused by antibiotic-resistant bacterial strains (Górski et al., 2007; Kutter et al., 2010), including bacteria resistant to multiple antibiotics (Górski et al., 2007; Hanlon, 2007). Moreover, it has been demonstrated that phage therapy is safe and relatively free of side effects (Borysowski and Górski, 2008; Łusiak-Szelachowska et al., 2014; Jończyk-Matysiak et al., 2015).

The use of bacteriophages in antimicrobial phage therapy is based on their ability to recognize, adsorb and multiply only within the bacterial cell and cause its lysis (Burrowes et al., 2011). Phage therapy is defined as the use of bacteria-specific viruses to combat pathogenic bacteria that cause infections (Loc-Carrillo and Abedon, 2011). Bacteriophages are usually highly specific and may be active against antibiotic resistant Gram-positive and Gram-negative bacteria (Biswas et al., 2002; Burrowes et al., 2011; Vinodkumar Srinivasa et al., 2011; Chhibber et al., 2013).

Phages have been used as a therapeutic agent in two main centers (Weber-Dąbrowska et al., 2000a,b; Sulakvelidze et al., 2001; Clark and March, 2006): in Poland [the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw (IIET PAS)] and at the Eliava Institute in Tbilisi, Georgia. In 2005 the Phage Therapy Unit was opened there – the first of its type in the European Union. Over the past few years, phage therapy has gained wider interest (Denou et al., 2009; Górski et al., 2009, 2012; Międzybrodzki et al., 2012; Pirnay et al., 2012; Henein, 2013; McCallin et al., 2013; Borysowski et al., 2014; Matsuzaki et al., 2014). Moreover phages are used as therapeutics also, e.g., in former USSR and the Russian Federation (Krylov et al., 2012; Krylov, 2014; Sarker et al., 2016).

SOURCES OF PHAGE ISOLATION AND PHAGE PROPAGATION HOSTS

The environment is a unique source of all types of phages, offering the possibility to isolate them for therapeutic purposes (Naghavi et al., 2013; Shukla et al., 2014). Phages are unevenly distributed, with the prime source of phages against human bacterial pathogens being the immediate hospital vicinity (Latz et al., 2016). For quality controlled production, phages active against *P. aeruginosa* and *S. aureus* were isolated from sewage and river water (Merabishvili et al., 2009). Already Dubos et al. (1943) isolated phages against *Shigella dysenteriae* 2308 from New York city sewage. The isolation of phages from environmental sources is more often possible when the sample contains the target bacterium (Gill and Young, 2011; Periasamy and Sundaram, 2013). An optimal source for searching for new phages is sewage (Łobocka et al., 2014; Weber-Dąbrowska et al., 2014). Vandersteegen et al. (2013) isolated raw inlet sewage water phages, Romulus and Remus, active against *S. aureus*. These phages infected 80% of tested strains and had the potential to destroy biofilm. It is also possible to obtain new virulent phages from stream water (Ul Haq et al., 2011). Sheep and cattle fecal samples were also a source of newly isolated phages.

The occurrence of phages against bacteria that are not abundant, in samples obtained from the environment, is usually low, so the simplest and most direct methods for their isolation may be unreliable. Therefore it is recommended to use large volume sample because concentration and/or enrichment methods may be relevant (Gill and Young, 2011). In larger sample volumes the possibility to detect phage is increased, and this type of searching may be recommended when too few desired phages are present in the sample (Gill and Hyman, 2010). Sometimes phage inactivation may occur during the waste water treatment process; e.g., during anaerobic-aerobic adsorption on solid particles as well as aerobic treatment using activated sludge or flocculants (Tanji et al., 2002). But, in Bacteriophage Laboratory IITD PAS phages active against *Enterococcus* and *Staphylococcus* strains were isolated from drinking water (Weber-Dąbrowska et al., 2014).

The broad host range phage isolation protocol from sewage and pond water was described by Jensen et al. (1998). The widely considered narrow host range of isolated phages may be the result

of the isolation procedure rather than the naturally occurring phage spectrum. Host specificity of phages that are isolated from aquatic samples is broader than that of phages isolated from other types of samples (Wommack and Colwell, 2000). Two staphylococcal phages, PhiSA039 and PhiSA012, isolated from a municipal wastewater treatment plant in Tokyo were shown to have a wide host range (Synnott et al., 2009). Similarly, the P-27/HP bacteriophage isolated from sewage was polyvalent and was able to control multidrug resistant *S. aureus* (Gupta and Prasad, 2011). It was able to kill 60% of isolates of *S. aureus* from humans. Parra and Robeson (2016) found three polyvalent bacteriophages (FC, FP, and FQ) infecting the *S. enterica* serovar Choleraesuis and *Escherichia coli* strains, in wastewater samples in Chile. Yu et al. (2015) suggested that polyvalent phages may be rapidly isolated from the environment by using different sequential hosts. Their novel method is based on two sequential multihost isolations, and both culture-dependent and culture-independent phage libraries were tested for phages of broad range of infectivity. These methods were found to be useful for isolation of broadly polyvalent phages that were able to infect bacteria of distantly related taxons. Another example of unexpectedly broad host-range phage is phage JHP, which was isolated recently by Khawaja et al. (2016). Unfortunately, it is difficult to obtain phages whose specificity is broad enough to cover all strains within a pathogenic species, despite the fact that it is possible to isolate phages with a broad host range (Nilsson, 2014). A typical problem in phage therapy is a narrow host-range of certain phages. T4 phages tested by Denou et al. (2009) for the efficacy in curing *E. coli* diarrhea proved to be not only species- but also strain-specific. Therefore, it was necessary to use phage cocktails containing 10 and 16 phages that cover half to two thirds of *E. coli* strains (representing the five main pathotypes isolated from patients suffering from diarrhea). Ready-made, broad-host-range cocktails that contain phages active against different pathogens may be a good solution when rapid phage application is needed.

The richest source of the most effective therapeutic phages is the material from patients who recovered from an infection (D'Herelle, 1938). Mattila et al. (2015) isolated phages on demand from sewage. They emphasized the ease of isolation of phages against such pathogenic strains as *Pseudomonas aeruginosa*, *Salmonella*, extended spectra beta-lactamase *E. coli*, and *K. pneumonia*, but greater difficulty to find phages active against vancomycin-resistant *Enterococcus* and *Acinetobacter baumannii* strains. Isolation of phages that are active against *S. aureus* turned out to be the most difficult. However, e.g., Kwiatek et al. (2012) and Sahin et al. (2013) obtained phages active against *S. aureus* clinical MRSA strains.

O'Flynn et al. (2004) isolated phages active against the *E. coli* O157:H7 strain from human fecal samples of patients with gastrointestinal disorders, and from bovine feces. Certain phages were isolated from human skin swabs, in particular from bacterial strains (Brown et al., 2016), e.g., phages specific to *Propionibacterium acnes*. Raw hospital sewage samples were the source of the phiAxp-3 lytic phage which was active against *Achromobacter xylosoxidans* A22732 (Ma et al., 2016). This phage was active against four A. *xylosoxidans* strains tested.

Sometimes, it is possible to isolate a phage against certain strain of pathogenic species and then adapt this phage to another host strain against which one cannot easily isolate new phages.

Typically, high titer lysates containing phages that can effectively infect certain strains of bacterial pathogens can be obtained by the propagation of these phages in cells of related strains. Sometimes, to avoid the risk of infection and to simplify the culture procedure, a non-pathogenic bacterial strain – called a surrogate strain – may be chosen for phage isolation and propagation (Gill and Hyman, 2010). For example, David et al. (1980) chose for their research *Mycobacterium smegmatis* – a species safer than the closely related pathogenic *M. tuberculosis* – to propagate the D29 phage. This strain grew rapidly compared to the pathogenic *M. tuberculosis* and was also susceptible to the D29 phage; therefore for practical reasons it was chosen for the propagation of this phage.

SYNERGY BETWEEN PHAGES

Some phage preparations are in the form of a cocktail – they may be composed of more than one phage. Phages in the cocktail may be active against various strains of the same bacterial species, but the killing of target bacteria by the cocktail may be more effective than expected based on the lytic activities of single cocktail phages. This phenomenon is referred to as synergy, and it may be helpful and useful from the therapeutic point of view. According to research conducted by Schmerer et al. (2014), it is possible to obtain such synergy if one phage can facilitate the infection of the same bacterium by another phage. They isolated the phage from sewage and observed that it caused the formation of large plaques on a mucoid *E. coli* strain. The large size of plaques resulted from the combined activity of two phages – J8-65 (turbid plaques with a halo effect) and T7 (forming small plaques). The combination of these two phages resulted in 10–100-fold higher killing efficacy of host bacteria compared to each phage alone. The likely cause of this synergy was the activity of J8–65 colanidase - an enzyme which degrades the exopolysaccharide of mucoid bacterial surface. By degrading a mucoid barrier protecting cells from the T7 adsorption, the colanidase provided easier access of T7 to the bacterial cell surface receptors. This observed synergy was temperature- and media-dependent. From a practical point of view, phage-encoded depolymerase enzymes may facilitate phage access to bacteria in a biofilm community (Cornelissen et al., 2011; Pires et al., 2016), which may have practical implications in biofilm treatment. Phage efficacy in destroying biofilm was observed in the case of biofilms formed by, e.g., *Enterococcus* or *Staphylococcus* (Gutiérrez et al., 2015; Khalifa et al., 2015).

The knowledge based on the possibility to obtain synergy may significantly improve production of phage preparations intended for phage therapy, as it strengthens their potential efficiency.

METHODS OF PHAGE ISOLATION

Samples that are believed to contain phages may be directly – after sterilization by filtration (0.22 μm) or after incubation

(enrichment method) – plated on a bacterial host lawn to search for plaques – the simplest method for visualization of phage occurrence. It is worth considering that for each phage and bacterium specific medium, growth and storage conditions should be adjusted and evaluated (Fortier and Moineau, 2009). Moreover, for new phage isolation it is recommended to use one bacterium in the exponential phase of growth (Wommack and Colwell, 2000; Kutter, 2009). To verify the findings, each test should be performed at least in duplicate.

Liquid samples seem to be easier for phage searching, whereas solid ones need to be suspended in a sterile liquid medium. Sometimes it is possible to use pooled samples (e.g., in searching for phages in the urine of an infected patient, when the material was collected at different time points). However, independent testing seems to be a more likely method of isolating phages than pooling (Gill and Hyman, 2010). The main steps in phage isolation for therapeutic purposes are presented in **Figure 1**. Identification of new phages and adding them to the collection begins with multiplying phages obtained from an environmental sample with the use of the target bacterial host and checking the activity of the phage against strains of a well-defined bacterial collection. This procedure is known as the determination of the phage host range on a target host strain panel (Mirzaei and Nilsson, 2015; Pirnay et al., 2015). Interestingly, some phages can form plaques that are too small to be seen. An example is *Bacillus* phage G, which cannot diffuse through agar, due to its extraordinary large virion size (Hendrix, 2008).

There are many ways to improve the visibility of phage plaques on a bacterial lawn. One possible method, especially in the case of phages isolated from environmental samples, is the use of sublethal doses of antibiotics (e.g., 2.5–3.5 μg/ml of ampicillin, depending on the top agar thickness), which was suggested by Łoś et al. (2008). This allows one to obtain plaques of increased diameter or obtain any kind of visible plaques in standard conditions in the case of *E. coli* phages. Similarly, Kaur et al. (2012) also used sublethal doses of antibiotics (inhibitors of proteins synthesis) to increase the size of MR-5 phage plaques (which are usually tiny, small, and difficult to visualize) on the methicillin-resistant *S. aureus* strain. The addition to the soft agar layer of 2,3,5-triphenyltetrazolium, sodium thiosulfate or ferric ammonium citrate, as it was done in the case of *Salmonella enterica* subsp. *enterica* bacteriophages isolated from swine lagoon effluent may be another way to facilitate plaque visualization (McLaughlin and Balaa, 2006).

For therapeutic purposes it is necessary to select a phage against the isolated bacterium that is the cause of infection. There may be a large number of phages in the tested sample, but only one may be identified on a particular bacterial strain lawn (Kutter, 2009).

When multiple phages are expected to be present in a tested sample, the enrichment method is used with more than one bacterium, and the obtained cell lysate may potentially be a source of many phages (Łobocka et al., 2014). In this method bacteria are usually cultivated in LB medium or in broth, sometimes in BHI broth (Beheshti Maal et al., 2015). D'Herelle (1938) recommended applying more than 50 bacterial strains in this procedure. One may even use bacteria from different taxons (genera, species) and obtain phages with a wide host range (Łobocka et al., 2014).

Liquid culture and the solid plate method are preferred for phage isolation. The double layer method is used to determine the titer of phage in the sample and/or preparation at each step of searching and preparing phage preparations used as therapeutics (Gratia, 1936; Adams, 1959). Fresh bacterial culture in the expotential phase of growth is mixed with the environmental sample sterilized by filtration (where phages are likely to be detected) and incubated properly depending on the host bacterial strain, to allow the phage to amplify and attain a high titer. If a plaque is observed on the bacterial lawn, the next step of phage isolation is to pick and transfer a single plaque into liquid medium (Mattila et al., 2015). The efficiency of plating (EOP) of some phages, e.g., the T4 phage, is 100%, which means that in optimal conditions every single particle of the phage can infect a host cell and produce plaques (Kutter, 2009). Typically, three to five passages are necessary to ensure the multiplication of a single phage from the environmental sample (Łobocka et al., 2014). The monoclonality of phage preparations is a separate issue, as commonly used phage propagation strains may be a source of contaminating undesired phages.

To detect the presence of phages in samples in which we could not detect them using the plate method, the colorimetric method is used in our laboratory (**Figure 1**). It is simple and sensitive, and it offers unequivocal results (McLaughlin, 2007; Żaczek et al., 2011). It exploits the ability of living bacterial cells to reduce the tetrazolium to formazan, which is visible as the appearance of a dark red color in the bacterial culture over time. Lysis of bacteria results in the lack of culture medium staining as compared to the control and may suggest the presence of phages in the tested samples. Advantages of this method include the possibility to detect phage particles present in the sample in a low number and the possibility to detect phage presence in many samples simultaneously during one experiment. Fischer et al. (2013) also recommended the microplate test for the rapid determination of bacteriophage susceptibility of *Campylobacter* strains. Moreover this method is suitable for automation of the phage susceptibility testing. Additionally, the microplate test allows one to examine a 5–10-fold greater number of isolated strains, compared to the conventional plate method.

A method that is useful for the acquisition of phages for cocktails with great therapeutic potential is so called step-by-step (SBS) method (Gu et al., 2012). It is based on the selection of lytic phages active against both the wild type phage-sensitive strain and its phage-resistant variant. The wild type bacteria and the first phage-resistant strain are used for isolation of the first and the second phage, and bacteria resistant to the second phage are applied to isolate the third phage.

Mirzaei and Nilsson (2015) compared the efficiency of plating (EOP) and host range of several phages using the spot test and the double-layer agar method that are commonly used in the initial phage characterization. Strains of the standard *E. coli* and *Salmonella* reference collections, and ESBL-producing *E. coli* served them to isolate phages from sewage water and waste water from Stockholm (including hospital samples). Six phages of the widest lytic spectrum were chosen for the comparative analysis

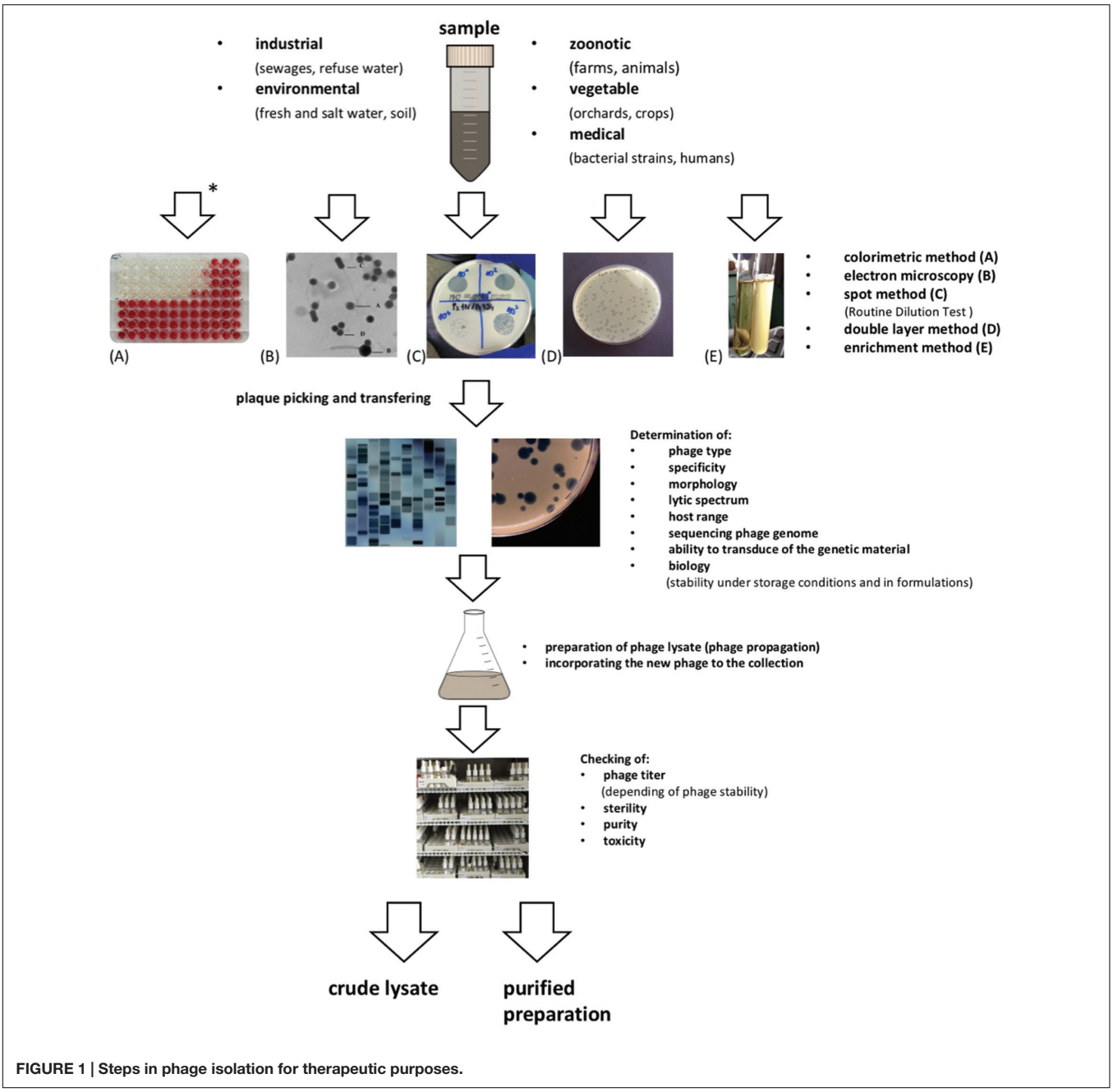

FIGURE 1 | Steps in phage isolation for therapeutic purposes.

of both methods. The results did not fully correlate. The authors suggested that the differences may be an effect of lysis from without, the presence of bacteriocins or endolysins in the lysate, or the presence in certain bacterial strains of prophages encoding phage resistance genes.

Most often, phage growth in laboratory conditions does not reflect its good propagation *in vivo* (Weld et al., 2004). Phages that have similar properties and may have a similar host range under laboratory conditions can have different therapeutic efficacy *in vivo*. Therefore, it is important to select for therapeutic purposes only phages that have high fitness *in vitro*, and try to simulate conditions corresponding to the site of infection to check phage potential therapeutic efficacy (Bull et al., 2010; Bull and Gill, 2014; Łobocka et al., 2014). It is believed that phage success in therapeutic application may result from appropriate phage-bacterial dynamics (Bull and Gill, 2014).

For phage screening it is recommended to use as many bacterial strains as possible. Phage typing and the host range are usually determined using a routine spot test, in which phage lysates or their dilutions are used. This method is simple, quick and not expensive, yet very useful, and it gives an overview of the bacterial sensitivity to a particular set of phages.

THERAPEUTIC PHAGES

The most common phage preparations are colloid suspensions (protein based), most often prepared freshly (Vandenheuvel et al., 2015). For therapeutic purposes bacteriophages should be dispersed in solution, stable, safe and have a high affinity to bacteria. Thus, lytic tailed phages belonging to the *Myoviridae*, *Siphoviridae,* and *Podoviridae* families are used for phage therapy (Wittebole et al., 2014).

The historical collection of Bacteriophages in the IIET PAS was initiated by Ludwik Hirszfeld, who was working with *Salmonella* bacteriophages. Currently, the collection contains approximately 800 phages, active against mainly such pathogens as *Escherichia, Morganella*, *Klebsiella*, *Enterobacter*, *Enterococcus, Pseudomonas, Staphylococcus*, and *Salmonella*, and it is being continuously expanded. In the collection there are phages that were isolated from hospital sewage, inland and marine waters, animal and human feces, water, soil, plant, and clinical materials, e.g., from patients with the diabetic foot syndrome. Our phage collection contains phages that may be active against both *K. pneumonia* PDR (resistant to carbapenems), *E. coli* O157:H7 and O104:H4 (which caused the death of more than 50 patients in Germany in 2011). The procedure of preparing phages against *S. aureus* and *P. aeruginosa* has been in our laboratory under patent protection since 2002 (EP1 406 642 B1, PL 19543781, US 7, 232564 B2).

We noticed that phages in our collection have wide lytic spectra. The ones with the widest spectrum are staphylococcal phages (mean = 95%); coliphages have mean activity against *E. coli* strains of 86% (including ESBL-positive strains); *E. faecalis* phages destroy approximately 80% of our strains; phages active against *Klebsiella* lyse 62% of our strains; whereas *P. aeruginosa* phages lyse 56%. In the case of some *Salmonella* serotypes we have phages with activity as high as 100%. We found that newly isolated bacterial strains are less sensitive to "historical" phages. This phenomenon has been observed especially in the case of *E. coli*, *K. pneumonia*, *P. aeruginosa*, and, on a lesser scale, in the case of *S. aureus* strains.

It is recommended to maintain ongoing monitoring of available phage collections and update phage cocktails with newly isolated and characterized phages, removing old and inactive ones (Gill and Young, 2011). Our laboratory's phage collection increases constantly with phages isolated from both environmental and clinical materials. There is a possibility as well to use a preparation from our repository and quickly apply it in a patient (Fortuna et al., 2008). Even when phage sensitivity is changing during phage therapy, it is possible to find a new active phage.

Plaque morphology is important for the selection of therapeutic phages. Only clear, non-turbid plaques without a halo effect should be classified as the effect of the lytic phage activity. The obtained plaques are a complex result of different phage features, growth phase of the bacterial host, diffusion of virions in agar plate, and inactivation of phage particles by debris of bacteria (Abedon and Yin, 2009; Gill and Young, 2011). Similarities in morphology between plaques may often result from similar properties of phages that infect related bacteria. Phage adsorption from the therapeutic point of view is effective when it is fast – typically 70% or higher in 10 minutes. According to Bull and Gill (2014), it is possible to predict phage success when the phage-bacterial population dynamics is known. Successful treatment requires phages of high lytic activity – a feature that is correlated with the phage burst size (Mirzaei and Nilsson, 2015).

After phage isolation it is necessary to characterize every new phage before incorporating it into the collection. It is important to test: the latent period, the burst size (Hyman and Abedon, 2009; Mirzaei and Nilsson, 2015), host growth, "phage lifestyle", the adsorption rate, multiplicity of infection (MOI), and stability in storage condition, morphology of plaques, phage morphology, ultrastructure, and taxonomy (Ackermann and Heldal, 2010; Gill and Hyman, 2010). Moreover, phages should be characterized by sequencing of the whole genome to exclude toxin genes, virulence factor genes, and genes responsible for lysogeny, and they should have low transducing potential (Łobocka et al., 2014). For the safety of phage application it is also necessary to remove prophages and certain other mobile genetic elements from phage propagation strains. The risk of, e.g., virulence genes' presence in phage genomes cannot be totally excluded for safety reasons (Vandenheuvel et al., 2015). So, every single isolate should be considered as an entity with individual properties and requirements. Quality requirements for the production of phage preparation intermediate or finished products should be provided in laboratories with specified air quality and cleanliness, which minimize the risk of its contamination, and the biosafety level depends on the type of bacteria that are used in preparing formulations (Pirnay et al., 2015).

The requirement for manufacturing the BFC-1 phage cocktail for clinical trials using good manufacturing practices (GMPs) was obvious (Merabishvili et al., 2009). All products that are used for the preparation of the cocktail were certified. The preparation was cleared of endotoxins. Pirnay et al. (2015) the quality and safety requirements for sustainable phage therapy products were published. They suggested that manufacture of products for phage therapy should be described in detail at each stage of the process in standard operating procedures (SOPs) and validated.

CRITERIA OF PHAGE SELECTION FOR THERAPEUTIC PURPOSES

Phage Properties

The most important criterion for selecting phages for therapeutic application is their specificity – called affinity (Ly-Chatain, 2014). This property is of great importance for phage therapy. Therefore, one should be certain that an isolated bacterium is sensitive to the selected phage, as it may limit the effectiveness of phage application (the use of preparations such as cocktails may improve phages' lytic spectrum) (Goodridge, 2010). To manage the problem associated with phage specificity it is advisable to use phage cocktails which have broader lytic activity against bacteria than single phages. Phages with broad spectrum-strain lytic activity should be selected. Phage specificity for certain species of Gram-positive and Gram-negative bacteria (including antibiotic-resistant strains, e.g., MRSA, VRE, ESBL-producing *E. coli*, and multidrug resistant *P. aeruginosa*) allows one to eliminate

pathogenic bacteria without affecting the natural microflora. There are suggestions that some phages show family rather than genus or species specificity (Gill and Hyman, 2010).

There are numerous criteria that allow us to classify phages as useful for therapeutic purposes, including efficacy of phage therapy and the possibility to avoid adverse effects (Denou et al., 2009). First of all it is postulated to use only lytic phages. Temperate phages often give visibly turbid plaques (Gill and Young, 2011). According to Ackermann (2005), it is estimated that approximately half of the phages isolated from the environment are temperate. It is important for consideration, because most therapeutic phages are isolated from environmental samples. The presence of prophages within a bacterial strain may cause lysogenic conversion, which undermines phage applicability in therapy. Therefore, phages that contain genes for lytic phage repressors, integrases or transposases should not be used for therapeutic purposes (Łobocka et al., 2014). It is especially important, because certain mutants of temperate phages may cause the formation of clear plaques (Weigle, 1953).

For safety reasons, every phage that has potential for therapeutic application should be sequenced. Especially the possibility of phage genome participation in horizontal gene transfer should be investigated (Pirnay et al., 2015; Davies et al., 2016). Phages have not been demonstrated to have adverse effects on eukaryotic cells, and they should be safe for humans and animals.

Phage Stability under Storage Conditions and Formulations

Phages are composed of protein structures, and therefore they may be instable in solution form (Vandenheuvel et al., 2015). It is recommended to adjust the type of storage of phages to their biology and properties. Most often, the best way of their storage is cooling. Sometimes it is possible to add substances that may enhance phage stability in water suspension, or to preserve by freeze-drying, spray drying, or encapsulation. Some phages are considered as stable when their titer does not significantly decrease for a few days, while others preserve their stability for years. In the case of preparations applied for therapeutic purposes, there are quality and safety requirements that storage conditions should be validated and monitored (Pirnay et al., 2015).

Phage storage should assure the stability of phage particles in the form and conditions in which the preparation is stored, but the form of application should also protect phage particles against losing their activity. For example, Cooper et al. (2014) tested a cocktail of three phages (phages GL-1, GL-$1_{2.5}$, and LP-M_{10}) active against *P. aeruginosa* in the form intended for nebulized formulation. The assessed phages retained their activity over 180 days at a storage temperature of 4°C as well as at room temperature, and they were successfully nebulized with little loss of infectivity. However, the endotoxin levels [as verified by the Limulus amoebocyte lysate (LAL) test] were considerably higher than the acceptable levels.

Phage particles' stability in a preparation intended to be used in humans may depend on the composition of the phage preparation. O'Flaherty et al. (2005) observed that the K phage active against *S. aureus* showed stability in bismuth-based cream and this cream exhibited bactericidal activity also several days after storage at room temperature. What is more, in a semi-solid preparation – a Cetomacrogol cream – at a concentration of 2.5×10^8 pfu/ml per gram, *Propionibacterium acnes* phages remained active for 90 days when the preparation was stored at 4°C in a light-protected place (Brown et al., 2016). A non-ionic cream base preparation was chosen because it excludes potential occurrence of interactions of ions with phage particles. These properties, especially stability and its easy-to-use form, assure this preparation's potential for topical treatment of acne infection. Esteban et al. (2014) stabilized the K-phage and delivered it via an oil-in-water nanoemulsion. Freshly prepared K/nanoemulsion exhibited greater bactericidal properties than freely suspended bacteriophage. The emulsions containing the K phage killed cells of three different strains of *S. aureus*. Similar observations were obtained for preparations that were stored at room temperature and at 4°C, for up to 10 days. It was observed that bacteriophage activity was enhanced when it was delivered via nanoemulsions. Tablets and powders containing phages may ensure the phage titer and its stability in low humidity conditions (Schade and Caroline, 1944c).

The shelf life of phage preparations should be monitored, as phages differ in their titer's stability under storage conditions, and its persistence depends on suspended media as well as on different physical and chemical external conditions (Jończyk et al., 2011). A good candidate for phage therapy is a phage that can easily propagate in both laboratory and industrial conditions and is characterized by stability in storage conditions. For some phages storage in lysate form at 4°C is required, as no significant loss of its titer is observed (Ackermann et al., 2004). For other phages better conditions that allow titer stability to be maintained may be freezing at −80°C with the addition of glycerol or freeze-drying. Golec et al. (2011) offered a reliable method of preservation of tailed phages. It involves storage of phages in the DNA form frozen inside the infected bacterial cells at −80°C without observing any significant decrease in phage and host viability. The authors suggested that this method may be useful for newly isolated phages that have an unknown structure and biology and whose propagation conditions have not been determined and optimized. They also recommend this method as suitable for phage preservation. Puapermpoonsiri et al. (2010) indicated that the secondary drying cycle following lyophilization is most important for maintaining phage stability. Phages active against *Pseudomonas aeruginosa* and *S. aureus* were encapsulated into biodegradable microspheres (for inhalation) based on emulsification and freeze-drying, and it was observed that only a partial loss of phage lytic activity took place, which may result from the exposure of the phage particles to the water-dichloromethane interface, with the lyophilization process itself having little effect (Puapermpoonsiri et al., 2009). Also, Alfahdel et al. (2011) used *S. aureus* phage lyophilization in a viscous solution of 1–2% (w/v) hydroxypropyl methylcellulose (HPMC) with/without the addition of 1% (w/v) mannitol, yielding nasal inserts composed of a highly porous leaflet-like matrix. Phages were homogeneously distributed in the dried matrix. Their titers

decreased 10-fold following lyophilization to 10^8 pfu per insert; and further 100- to 1000-fold loss of pfu was observed during 6–12 months of storage at 4°C. It is possible to add substances, e.g., albumin, salts, or gelatin, to improve stability; but in the case of phages in pharmaceutical application it may be problematic (Gill and Hyman, 2010). Tovkach et al. (2012) prepared an *Erwiniophage* ZF40 suspension with the addition of a magnesium and gelatin STMF buffer which ensured the stability in long-term preservation of unstable enterobacterial phages (+4 to −2°C). The authors mentioned that the possible problems with long-term storage may be the result of phage virions' or DNA structural instability. Schade and Caroline (1944b,c) tested the influence of lyophilization on phage stability. They observed that that addition of, for example, yeast extract, lecithin, and raw egg white, protected dysentery phage Sh-1, promoting the stability of this sensitive phage during lyophilization. Interestingly, the dysentery phage was protected against damage that could be the result of lyophilization, and obtained powders were stable during 12 months of storage over a desiccant at 37°C (Schade and Caroline, 1944a). Also phages active against *S. aureus*, including MRSA strains, were lyophilized with the presence of different stabilizers: sucrose, trehalose, mannitol, and glycine, polyvinylpyrrolidone and PEG 6000 (polyethylene glycol) at different concentrations (Merabishvili et al., 2013). Trehalose and sucrose (0.5 M) were found to be the best additives protecting the ISP phage, whose titer dropped by 1 log after lyophilization and by another one after being stored in powder form for 37 months at 4°C.

Fortier and Moineau (2009) stated that lyophilization is a more effective method to preserve phage stability than storage in a solution, and lyophilized phages had higher heat stability and were resistant to drying. As they observed, phage protective action was provided in egg yolk. Other researchers observed that storing the BFC-1 phage cocktail for 12 months at 4°C, did not cause the observed loss of stability (Merabishvili et al., 2009).

Importantly, inactivation of phage particles may occur before reaching the site of infection. This situation should be possible to avoid; therefore the initial dose and the route of phage administration should enable them to circulate, achieve the infection site and multiply there as long as pathogenic bacteria are present (Gill and Young, 2011). However, suspensions of phages that exceed 10^{13} pfu/ml seem to be difficult to obtain since concentrated suspensions become too viscous (Nilsson, 2014).

Phage preparations that are used as therapeutics may contain only one phage or be a cocktail consisting of two or more phages. The latter type of preparation's composition may prevent the occurrence of cross-resistance, and, based on this phenomenon, a bacterium which is resistant to one phage may remain sensitive to another, and cocktails that contain phages using different receptors for binding to bacteria may be a better solution for eliminating the development of resistance in bacteria (Gill and Hyman, 2010). Some changes in lytic patterns of patients' strains isolated during the course of phage therapy may be observed. It may be due to instability and heterogeneity of bacterial strains as well as different bio- and serotypes of bacteria or changing phage receptors. According to Chan and Abedon (2012), the treatment of infections caused by antibiotic-resistant bacteria seems to be more effective when a phage cocktail is used, compared with single phage efficacy.

LIMITATIONS OF PHAGE PROCUREMENT FOR THERAPEUTIC PURPOSES

The most important factor for effective use of phages as therapeutics is the widest possible knowledge (Goodridge, 2010) regarding biology of phages, their individual properties and stability in different forms of available preparations (both lysates and purified preparations). There are opinions that phage treatment may induce the production of antiphage antibodies and this may limit the phage antibacterial activity (Sulakvelidze et al., 2001). Moreover, it is possible that bacteria may develop resistance to phages. The next limitation is the lack of legislative solutions adapted to broad use of bacteriophages to combat antibiotic-resistant bacteria.

The presence of phages of a wide host range in the collections, on one hand, allows the number of phages needed in a collection to be reduced, and on the other hand, it offers the opportunity to increase the probability of finding phages active against emerging drug-resistant bacterial strains (Mirzaei and Nilsson, 2015). The phage searching and isolation procedure should be designed in such a way as to prevent possible treatment failure resulting from wrong phage selection (Gill and Hyman, 2010).

Clear lysis zones observed on a bacterial lawn are most often the result of phages' lytic activity. Sometimes, it is possible for a phage preparation to contain a factor that causes lysis but it is not directly connected with phages' lytic activity (Hyman and Abedon, 2010). The agents responsible for the observed effect could be bacteriocins, endolysins (that may cause lysis from without). Additionally, abortive infection can cause bacterial death without the release of new phage progeny. Interestingly, filamentous phages form plaques, but they do not lead to the lysis of bacterial cells (Gill and Hyman, 2010). Also *Bdellovibrio* bacteria can form plaques (Hobley et al., 2006).

It is extremely important to keep in mind that there are differences between phages themselves, and the type of their isolation, tested material, and thus the type of procedure during phage propagation should be adjusted to an individual phage and should depend on the type of host bacteria in which phages are propagated (Mattila et al., 2015). Sometimes it is not possible to detect any plaques on the bacterial lawn. The reason for this phenomenon may be that the bacterial host is insensitive to the phage, and it does not indicate that there is no phage in the sample but that the wrong bacteria were chosen (Gill and Young, 2011) or that phage particles could disintegrate (Gill and Hyman, 2010). One of the isolation limitations may result from the differences in propagation ability of phages under experimental conditions or from the inability to isolate phages which are present in the sample (Hendrix, 2002). Bacteria that are present in environmental samples are unculturable (we cannot isolate them, or we do not know the culture requirements) in laboratory conditions with commonly available equipment and methods, and therefore we are able to isolate only a small

number of phages occurring in these sources. Cultures used in the search for phages occurring in water samples should be incubated at 25°C rather than 37°C. Interestingly, it is of great importance to adjust proper culture conditions to a particular bacterial host's requirements. Procurement of phages generally may seem to be easy, but matching them for therapeutic purposes should be adjusted to individual phage properties. Other limitations may be due to a large number of genetic changes in bacterial and phage strains. This phenomenon may be in part a result of the acquisition of resistance to phages by bacteria.

Phage virions within the mammalian body are exposed to factors that may cause their inactivation. Contact with the acidic gastric pH (Verthé et al., 2004), alkaline bile salts (Ma et al., 2008), antibodies (Keller and Traub, 1974) and phagocytes, as well as the toxicity of urea, may result in the loss of their lytic activity. In order to avoid adverse factors, searching for improved methods of administration and formulations for new forms that reduce the exposure of phage to destructive conditions is desirable. For example, the encapsulation of phages may enable phage particles to retain the activity in unfavorable conditions (Dini et al., 2012; Colom et al., 2015).

Therapeutic phages are currently applied in the form of lysates or purified preparations, and they cannot be toxic (Skurnik and Strauch, 2006; Skurnik et al., 2007). Due to the necessity to keep the final preparations safe, it is essential that the media used for phage cultivation and propagation are definitely free from such dangerous contaminants as prions, viruses and allergens (Goodridge and Abedon, 2008).

In order to prevent an anaphylactic reaction in response to bacterial components, such as endotoxins (which may be a component of the phage lysate), some investigators recommend the use of purified preparations (Skurnik and Strauch, 2006; Loc-Carrillo and Abedon, 2011). However, while it is thought that the staphylococcal phage lysates stimulate the body's immune response, purified preparations may show immunosuppressive activity (Górski et al., 2012). Purification of phage preparations of bacterial endotoxins is becoming more effective. Endotoxin levels in preparations for intravenous use should not exceed 5 endotoxin units (EU $1 \approx 100$ pg) per kilogram of body weight per hour (Wanninger and Artz, 2009). The most common method uses isopycnic density gradient centrifugation. Sometimes this method may be problematic – phage particles may be damaged by high salt concentrations (Gill and Young, 2011). There are methods, e.g., ion exchange chromatography or affinity chromatography (Oślizło et al., 2011; Narasimhaiah et al., 2013), which allow one to obtain purified preparations with reduced endotoxin content. Other methods used for phage purification may be cesium chloride (CsCl) gradient centrifugation or sucrose gradient (Kleiner et al., 2015). The yield of phages using the CsCl method is low, and some phages may be lost due to aggregation or damage of virions. High performance liquid chromatography (HPLC) may also be applied successfully to concentrate and purify tailed phages (Adriaenssens et al., 2012). Both the methods of staphylococcal phage purification (P391328) and the method of obtaining bacteriophage preparations containing trace amounts of endotoxins (P382800; US 20100227376 A1) have been used in our laboratory under patent protection. For phage purification, Merabishvili et al. (2009) recommended the use of the Endotrap blue chromatographic column that binds endotoxins.

MODIFIED PHAGES

Apart from isolating naturally occurring phages from environmental samples, it is possible to obtain genetically modified phages (by recombination of phage genomes, site-directed mutagenesis, selection of spontaneous mutants or phage display methods). These methods offer a great therapeutic tool for dealing with the challenges of phage therapy (Moradpour and Ghasemian, 2011; Chhibber and Kumari, 2012; Dąbrowska et al., 2014). Modified phages may be deplated of undesired properties, may have changed specificity or improved therapeutic potential, as e.g., phages that have dual activity – antitumor and bactericidal – without an observable reduction in antimicrobial activity (Dąbrowska et al., 2014). These findings open new possibilities for the use of phage therapy.

The discovery of bacterial adaptive immunity system, CRISPR/Cas (Clustered Regularly Interspaced Palindromic Repeats [CRISPR]/CRISPR-associated [Cas]) systems, has opened new possibilities in the design of a genetic load that can be delivered by bacteriophages. In nature, bacteria can acquire resistance to a bacteriophage by the incorporation of the genome fragment of this bacteriophage as a spacer between repeats in the CRISPR locus (Szczepankowska, 2012). In the case of the CRISPR/Cas systems of type II, small CRISPR RNAs (crRNAs) produced by the processing of CRISPR locus transcripts serve to guide a nucleolytic enzyme to target invading phage or other DNA molecules, if they are only complementary to them. The CRISPR/Cas systems were also found in some phages (Hargreaves et al., 2014; Zheng et al., 2016). A CRISPR/Cas system that is encoded by a virulent *V. cholerae* phage is used to destroy the DNA of its host chromosomal island, which excises from the chromosome in response to phage infection, circularizes and interferes with phage development (Seed et al., 2013). This natural system provides a proof of the concept that phage-mediated delivery of CRISPR/Cas-encoded RNA-guided nucleases (RGNs) is an efficient way to target bacterial DNA in a sequence specific manner. Indeed staphylococcal ϕMN1phage particles packed with engineered phagemid DNA carrying CRISPR/Cas with spacers targeting certain *S. aureus* virulence genes killed virulent, but not avirulent *S. aureus* (Bikard et al., 2014). They could also reduce the number of virulent *S. aureus* strain cells on a skin of infected mice. Modified phages with multiple varying CRISPR spacers were effective in destruction of multiple DNA targets in the infected bacterial cell.

Phages of changed properties may be also obtained by chemical modifications. Modifications of phage particles by attaching monomethoxy-polyethylene glycol (mPEG) to proteins caused the phage to become less immunogenic. The PEGylated phage caused a decrease in the level of cytokines such as IFN-γ and IL-6 in both the non-immunized and the phage immunized mice (Kim et al., 2008). The modified phage particles were

characterized by a longer half-life in the organism, which may be of practical importance in enhancing the effectiveness of phage therapy. Our recent hypothesis suggests that it might be possible to obtain modified phages with homing peptides that enable phage localization in infected tissues (Górski et al., 2015). Taken together, all kinds of phage modifications open new possibilities for the use phages in antibacterial therapies.

CONCLUDING REMARKS

Phage therapy offers a real chance for patients suffering from infections caused by antibiotic-resistant bacteria. Widening our knowledge about phage biology, their individual properties and stability in different forms of available preparations offers the most significant prospect for effective application of phages as therapeutics (Goodridge, 2010).

Phage searching and isolation procedures should be adjusted to the type of bacterial strain being the host of the phage, which would also prevent treatment failures that may lead to phage misselection. Phage preparations intended for treating infections in humans should be safe, sterile and endotoxin-free. What is more, the methods of administration and formulations for new forms that reduce the exposure of phages to destructive conditions should be improved. Genetically modified phages with broader activity may be obtained, and it is possible to shape their activity depending on the therapeutic needs. The progress in the knowledge about phage genomics, immunobiology and experimental therapy in animals and in humans suggest that phages could become the antibacterial drugs of the 21st century.

AUTHOR CONTRIBUTIONS

BW-D and EJ-M drafted the main part of the manuscript. MŻ performed part of manuscript and the **Figure 1**, MŁ and MŁ-S prepared parts of manuscript. AG gave support and conceptual advice at all stages of manuscript preparation. All authors revised the manuscript.

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BW-D and AG are inventors of a patent (EP1 406 642 B1, PL 19543781, US 7, 232564 B2) for procedure of preparing phages against *S. aureus* and *P. aeruginosa* (P382800; US 20100227376 A1) and a method of obtaining bacteriophage preparations containing trace amounts of endotoxins owned by the Institute of Immunology and Experimental Therapy. EJ-M, MŁ, MŻ, MŁ-S, declares no potential conflict of interest.

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On-Demand Isolation of Bacteriophages Against Drug-Resistant Bacteria for Personalized Phage Therapy

*Sari Mattila, Pilvi Ruotsalainen and Matti Jalasvuori**

Department of Biological and Environmental Science, Centre of Excellence in Biological Interactions, University of Jyväskylä, Jyväskylä, Finland

Bacteriophages are bacterial viruses, capable of killing even multi-drug resistant bacterial cells. For this reason, therapeutic use of phages is considered as a possible alternative to conventional antibiotics. However, phages are very host specific in comparison to wide-spectrum antibiotics and thus preparation of phage-cocktails beforehand against pathogens can be difficult. In this study, we evaluate whether it may be possible to isolate phages on-demand from environmental reservoir. We attempted to enrich infectious bacteriophages from sewage against nosocomial drug-resistant bacterial strains of different medically important species in order to evaluate the probability of discovering novel therapeutic phages. Stability and host-range were determined for the acquired phages. Our results suggest that on-demand isolation of phages is possible against *Pseudomonas aeruginosa, Salmonella* and extended spectrum beta-lactamase *Escherichia coli* and *Klebsiella pneumoniae*. The probability of finding suitable phages was less than 40% against vancomycin resistant *Enterococcus* and *Acinetobacter baumannii* strains. Furthermore, isolation of new phages against methicillin resistant *Staphylococcus aureus* strains was found to be very difficult.

Keywords: antibiotic resistance, ESBL, MRSA, phage therapy, phage cocktails, bacteriophages

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****Correspondence:***
Matti Jalasvuori
matti.jalasvuori@jyu.fi

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INTRODUCTION

Antibiotic resistance is an emerging global health crisis, resulting from the continuous use (and misuse) of antibiotics in healthcare, farming industry, and elsewhere (Cantas et al., 2013; World Health Organization [WHO], 2014). Phage therapy refers to the utilization of bacteriophages (or just phages, viruses infecting bacteria) to treat bacterial diseases (Abedon et al., 2011). Given the increasing number of drug-resistant bacterial infections, especially within hospital settings, the exploration of alternatives to conventional antibiotics has become an important research objective (Finch, 2011; Sommer and Dantas, 2011). Bacteriophages are very abundant (Hendrix et al., 1999) and every bacterium is likely to have their own specific viruses that could be utilized as antibacterial agents (Clokie et al., 2011; Flores et al., 2011; Örmälä and Jalasvuori, 2013). Historically, phages were used therapeutically already in the early 20th century (Sulakvelidze et al., 2001). Yet, the discovery of broadly effective antibiotics led to the demise of the development of phage therapy in western countries and only as the antibiotics are starting to fail there has been a serious attempt to restore the old tool. However, the second coming of phage therapy faces challenges regarding

to the strict regulatory guidelines and the development of effective therapeutic practices (Gill and Hyman, 2010; Lu and Koeris, 2011; Keen, 2012). Yet, phage therapy can provide an evolutionarily sustainable alternative to conventional antibiotics, should we be able to adjust our regulations and procedures to meet the special requirements of phage based medicine (Keen, 2012; Örmälä and Jalasvuori, 2013).

It is important to note that phages infect bacterial hosts very selectively. Often, the narrow host-range is considered as an advantage over traditional antibiotics since phage treatment can focus accurately on the pathogen without harming commensal bacterial flora (Loc-Carrillo and Abedon, 2011). On the other hand, bacteria develop resistance also to phages rapidly, and thus the achieved antibacterial effect may be transient (Hyman and Abedon, 2010; Labrie et al., 2010). When multiple different phages are used simultaneously in a phage cocktail, development of resistance is less likely (Skurnik et al., 2007; Chan et al., 2013). However, it is challenging to obtain a set of phages that is effective against all variants of a given pathogen (Pirnay et al., 2011; Chan et al., 2013). There can be a tradeoff between the host range and the therapeutic efficacy of a cocktail for a specific species of bacteria: when the number of phages in a cocktail increases in an effort to increase the host range of the cocktail, the number of phages against a specific strain of bacteria may decrease. Therefore, the host specificity of phages, while in theory beneficial, poses a practical problem when combined with the rapidly emerging resistant phenotypes.

In principle, it is possible to acquire bacteriophages on-demand to treat, for example, infections that are resistant to all known antibiotics and off-the-shelf (standardized) phage-therapy products (Keen, 2012; Örmälä and Jalasvuori, 2013). Tailoring a therapeutic cocktail personally for each patient would allow the cocktails to comprise phages that are effective against the bacterial strains responsible of the infection (Pirnay et al., 2011; Chan et al., 2013). Therefore and in comparison to premade cocktails, a personalized phage therapy does not carry a surplus of ineffective phages. Indeed, there are older studies suggesting that tailored phage treatments are several times more effective compared to standardized cocktails (Zhukov-Verezhnikov et al., 1978), and thus effective phage-therapy practices to treat constantly changing bacterial pathogens may depend on the adjustment of the treatment to the causative agent (Keen, 2012).

Generating a personal set of phages requires that the pathogen is isolated and, then, effective bacteriophages obtained against it. One possible way for identifying suitable viruses is to have a variety of bacteriophages isolated and prepared beforehand and then the causative pathogen screened through the phage-library (Chan et al., 2013). Alternatively, phages may be isolated as needed from environmental reservoirs. In some cases, the latter option may be inevitable due to the lack of infectious phages in the premade libraries against all possible bacterial variants. Ultimately, environment serves as the only source of practically endless phage variety and thus exploitation of the environmental resources forms the basis for personalized phage medicine.

While phages are known to be abundant, it is obvious that all environments cannot contain infective phages against all different bacterial hosts (see e.g., Flores et al., 2011; Atanasova et al., 2012). To the best of our knowledge, the probability of finding therapeutically useful phages against different resistant pathogens on-demand has not been studied *per se* despite the fact that it is likely to be the limiting factor in attempts to update premade cocktails or to generate on-demand personalized therapies (Chan et al., 2013). As an example, hospital acquired wound infections have been suggested to be especially suitable target for phage therapy as the causative agents are generally resistant to various antibiotics (Loc-Carrillo et al., 2012). Yet, there might be multiple different bacterial species present in these infections, including, e.g., *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Acinetobacter baumannii* (Agnihotri et al., 2004). Therefore, a successful phage-based treatment can be dependent on the practicality of being able to simultaneously and rapidly isolate new durable phages against very different pathogens.

In this study, we provide an evaluation of the on-demand isolation of phages against the most common hospital borne resistant pathogens: methicillin resistant *S. aureus* (MRSA), extended spectrum beta-lactamase (ESBL) *E. coli* and *K. pneumoniae*, multi-drug resistant (MDR) *P. aeruginosa*, vancomycin resistant *Enterococcus* (VRE), *A. baumannii* and different *Salmonella* species. All aforementioned species are also listed in CDC's report on the top 18 drug-resistant threats to the United States in 2013 (CDC, 2013). These bacteria commonly cause infections of skin, lung and urinary tract, as well as foodborne infections among others and affect people all around the world disregarding their background (CDC/FDA/NIH, 2011).

Sewage is known to be an optimal resource of phages (Lobocka et al., 2014), thus a wastewater treatment plant in Jyväskylä, Finland (Nenäinniemi) was used as the environmental reservoir for phage hunt. The stability of the acquired viruses and their cross-infectivity on other potential host strains were determined.

We demonstrate vast differences in probabilities of finding novel phages against different hosts by using enrichment method for isolation. There appears to be severe constraints in isolating phages on-demand against pathogens like MRSA. On the other hand, it seems feasible to obtain phages against ESBL positive *E. coli* and *K. pneumoniae* as well as *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria Strains and Culturing Conditions

Bacterial strains used in this study were mostly purchased from Medix Laboratories or acquired from Turku University Hospital (Supplementary Table S1). One *Klebsiella* strain and four *Enterococccus* strains were obtained from commercial culture collections. Aside from six bacterial strains, all had caused (antibiotic resistant) human infections and thus they represent pathogens that could have been treated with phages. Overall, we obtained 12 MRSA strains, 16 *E. coli* ESBL strains, 6 *K. pneumonia* (ESBL) strains, 17 *P. aeruginosa* MDR strains, 9 *A. baumannii* strains, 10 *E. faecium* (VRE) strains, 4 *Enterococcus faecalis* (VRE) strains, and 9 different *Salmonella* strains. Detailed

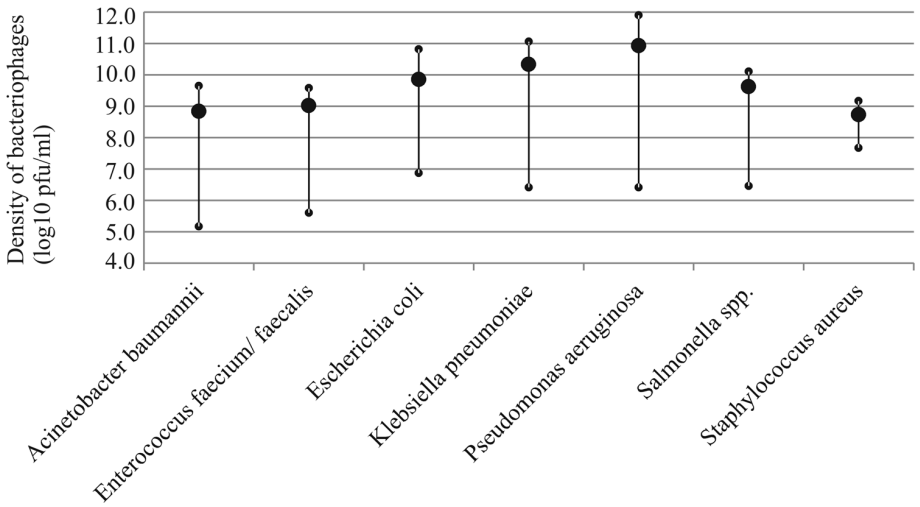

FIGURE 1 | Average density of infectious bacteriophage particles in the prepared stocks of each host species (large circle). Small circles indicate the maximum and minimum values observed.

TABLE 1 | Summary of the decrease in phage titers as observed after 1-month storage at +4°C.

Host bacterium	Average decrease in titer (log10)
Acinetobacter baumannii	0.973
Enterococcus faecium/ faecalis	0.222
Escherichia coli	0.496
Klebsiella pneumoniae	0.594
Pseudomonas aeruginosa	0.437
Salmonella sp.	0.529
Staphylococcus aureus	0.491

characterization of the bacterial strains was beyond the scope of this paper.

All bacteria were cultured in Lysogeny Broth (LB) -medium (Sambrook et al., 1989) at +37°C shaken 230 rpm (*Enterococcus* strains were cultivated without shaking).

Isolation Protocol

The following isolation protocol with slight modification in individual experiments was used throughout the study. Either unprocessed sewage samples or supernatants of turbid samples (centrifuged 3000–6000 *g* in Megafuge 1.0R, Heraeus, or in Eppendorf centrifuge 5702 R, 10–15 min at +4°C) were used in the enrichment steps. In cases where previous isolation attempts had failed to yield phages, the supernatant was also filtrated through a 0.45 μm filter to remove all remaining bacterial cells. The first enrichment step was conducted using 20–30 ml of sewage water filled up to 30–40 ml with LB-broth, depending on the volume of collected sewage samples. The target bacterial strain was added (50–200 μl o/n culture grown in LB-broth, 300 μl in case of *E. faecium* and *E. faecalis*) to enrich (potential) phages in the sample. These enrichments were cultivated overnight at +37°C, shaken 230 rpm. Bacteria from this enrichment culture were removed by centrifugation (3000–6000 *g* in Megafuge 1.0R, Heraeus, or in Eppendorf centrifuge 5702 R, 15–20 min, +4°C) and filtration (0.2 or 0.45 μm filter). The amount of potential phages in a 2.5 ml sample of the bacteria-free enrichment were further amplified by adding 2.5 ml of LB-broth and 50–100 μl of the target host bacterium and were grown overnight as above. The sample from this second enrichment step was centrifuged at 13 000 *g* for 15 min at room temperature and at least 10 μl the supernatant was plated on a LB-agar containing petri dish along with 100–300 μl of the host strain and 3 ml of melted 0.7% soft-agar. The plates were incubated overnight at +37°C. If plaques were observed on the bacterial lawn, a separate plaque was picked and transferred into 500 μl of LB-broth. A sample from this plaque-stock was further plated on the same host strain. Plaque-purification was performed three times for all discovered phages in order to isolate a single homogenous phage from the potentially heterogeneous phage mix that may have been present in the initial enrichment.

Due to poor isolation success for *S. aureus*, different modifications of the above-described method were used for enriching phages. The volume of the first enrichment step as well as the number of enrichment steps was increased (120 ml sewage sample + 70 ml L broth + 1 ml host overnight cultures in the first step). Rotation speed during shaken cultivation steps was varied between 100, 120, 180, or 360 rpm. In addition, samples from different sources were used for phage enrichment (River in Ljubljana, Slovenia, a water-lock sample from the Helsinki university hospital and soil samples from a livestock farm). These samples were not included in analysis of isolation success from sewage.

Preparation of Phage Stock

Semi-confluent plates (i.e., plates of which about half of the area is covered by phage induced plaques and the rest is bacterial lawn) were prepared by plating 100 μl of host strain (300 μl

TABLE 2 | Probability for discovering a bacteriophage from a sewage sample against different pathogens.

Bacterial pathogen	Mean hit %*	Isolation attempts	Number of strains hit
Acinetobacter baumannii	38.9	34	5/9
Enterococcus faecium/faecalis	33.9	27	5/14
Escherichia coli	90.6	35	15/16
Klebsiella pneumoniae	83.3	15	6/6
Pseudomonas aeruginosa	79.4	44	15/17
Salmonella sp.	88.9	11	8/9
Staphylococcus aureus	6.1	117	1/12

*As calculated over the bacterial strains of the given species.

of *Enterococcus* strains) and 3 ml of melted soft-agar with appropriate dilution of the phage stock. Plates were incubated overnight at +37°C. The soft-agar layers of semi-confluent plates were combined with 2.5–5 ml of LB-broth/plate. The combination was incubated for 4 h at +37°C, 230 rpm, and centrifuged at 6000 *g* for 15 min at +4°C (Megafuge 1.0R, Heraeus). If we were unable to get semi-confluent plates, we used as a combination "over-infected" plates supplied with 100–700 μl of the overnight-cultivated host strain. The supernatant was filtered (0.2 μm filter) and stored at +4°C.

Cross Infection Tests

All phages were used to cross-infect all different bacterial strains of its original host species (excluding *P. aeruginosa* phages as only half of them were used) for preliminary evaluation of their host range. Cross-infection tests were done by spotting 8 μl of phage stock dilution (1:10 or 1:100) on 100 μl bacterial overnight culture in soft-agar (0.7%). Plates were incubated at +37°C overnight. Formation of less opaque spots on the bacterial lawn was scored as a successful infection.

Phage Stock Stability

The titer of each phage stock was determined by standard double agar overlay method by plating a dilution series (10^{-2}–10^{-8}) immediately after preparation of the stock. Titer of the stock was determined again after 1-month storage (+4°C) to estimate the stability of the stock in LB-medium.

RESULTS

We evaluated the feasibility for generating a personalized phage-product on-demand against different bacterial pathogens. We chose bacterial species from seven different genuses that are responsible for the majority of hospital acquired bacterial infections, namely *Escherichia*, *Salmonella*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*, *Enterococcus,* and *Acinetobacer*. Total of 283 phage isolation attempts were conducted for 83 different host strains. Overall 108 bacteriophages were discovered. All of these viruses were characterized for their plaque morphology and stability (described individually for each virus in Supplementary Table S2).

Phages were isolated via three consecutive plaque-picking steps to avoid mixed-culture stocks. Due to different plaque morphologies and titers, the preparation of phage stocks was adjusted for each phage. However, no actual optimization of phage production was carried out. The density of viable phage particles was measured immediately after the preparation of the stock (**Figure 1**). In order to determine their viability for acute use, the number of viable particles was re-measured 1 month later (see summary in **Table 1**). On average, the titers of the stocks decreased around 0.5 log10 during the 1-month storage in L-broth in 4°C. However, for some phages of *Enterococcus*, the titers could no longer be resolved. Phage-specific titers and plaque morphologies are listed in Supplementary Table S2.

The probability for finding an infectious bacteriophage from sewage for different host bacterium varied substantially (**Table 2**). Namely, phages for only a single *S. aureus* strain, SA10, were discovered in total of 117 enrichment attempts (the phages specific to the one *S. aureus* strain were obtained at the same time and they produced visually identical plaques, thus we selected only one of these phages for subsequent analyses). Conversely, almost every isolation attempt yielded a bacteriophage for *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *Salmonella* strains. Phage isolation for *Acinetobacter* and *Enterococcus* had success rates between 30 and 40%. Given the medical importance of MRSA, we decided to investigate whether alternative source materials would be more suitable for discovering phages. We obtained water samples from a water lock situated in a room used to treat MRSA-patients in Helsinki University Hospital. Two phages for a single strain (SA10) were found from these samples. A single MRSA-specific bacteriophage was isolated from a set of soil samples acquired from a livestock farm. Also, a water sample from river Ljubljana, Slovenia, produced a single bacteriophage for strain SA10. Yet, we failed to find a single phage for any of the ten other MRSA-strains used in the isolation attempts.

As presented in **Figure 2**, we studied the host-range of the obtained phages in order to determine their cross-infectivity and thus the potential to combine previously isolated phages into phage-cocktails. Aside from a couple of exceptions, almost all phages isolated for any given *P. aeruginosa* strain could also infect majority of the other strains. However, we neither found any phages for strain PA15 nor did any of the other phages infect this strain. In addition, only 4 out of 20 tested *Pseudomonas* phages infected strain PA6. Detailed characterization of these particular strains was beyond the scope of this paper.

Along with *Pseudomonas* phages, some of the *Salmonella* phages had a wide host range. *E. coli* phages tended to infect more than one strain, except EC1P1, EC11P2, EC15P2, and EC16P1. For other bacterial species, isolated phages generally had less

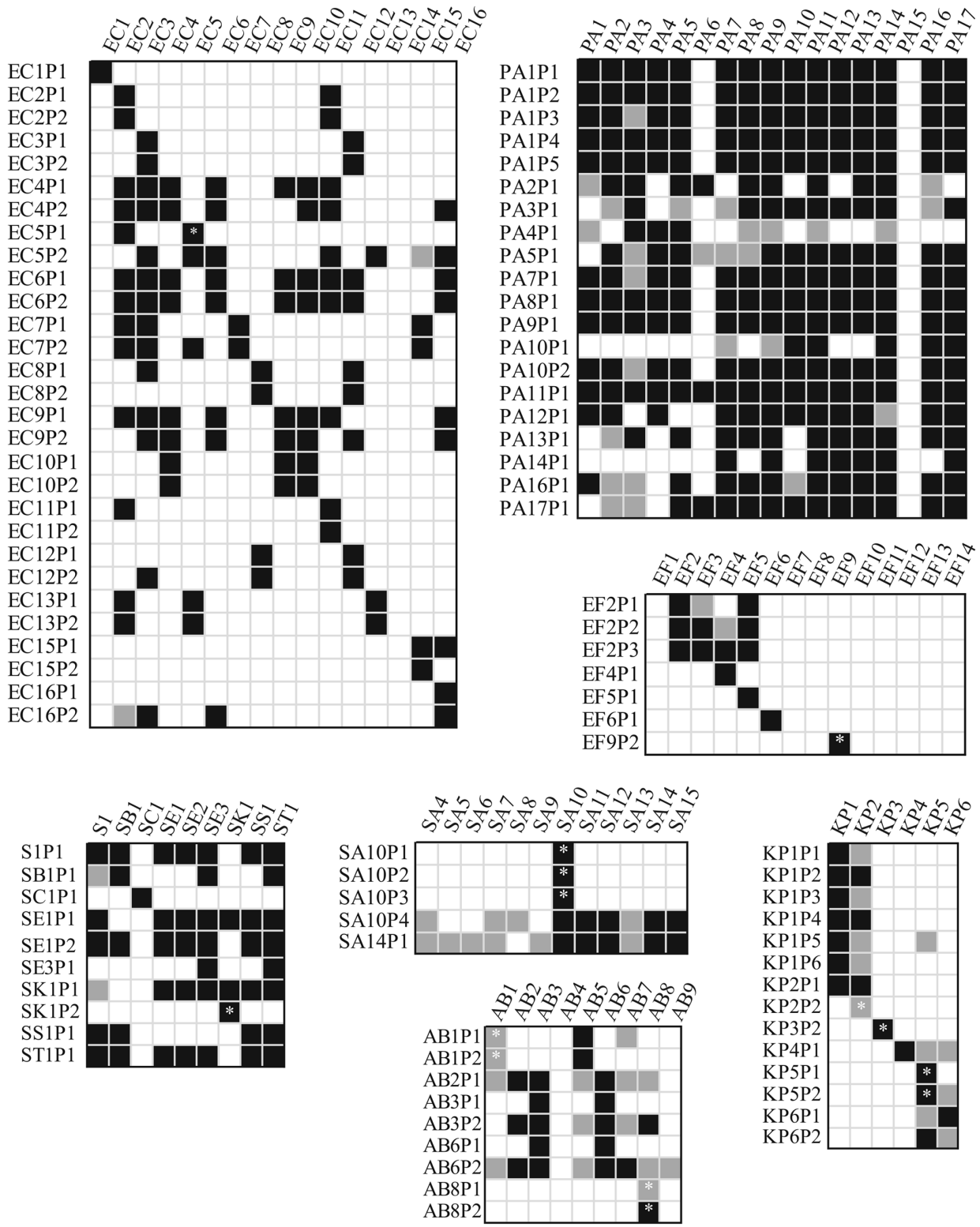

FIGURE 2 | Cross-infectivity of the isolated bacteriophages. Measurements where less than 10^5 pfu/ml were used are indicated with an asterisk. Only half of the isolated *Pseudomonas* phages were used in the experiments. White background indicates no lysis area, black marks clearly detected lysis area and light gray indicates very dim lysis area in the spot test. AB, *Acinetobacter baumannii*; EC, *Escherichia coli*; EF, *Enterococcus faecium* or *faecalis*; KB, *Klebsiella pneumoniae*; PA, *Pseudomonas aeruginosa*; S, *Salmonella* sp., and SA, *Staphylococcus aureus*.

alternative hosts, if any, indicating that a rapid preparation of a personalized phage-cocktail is likely to require multiple separate but simultaneous phage enrichments using a single bacterial strain as a host. Especially, *Klebsiella* and *Enterococcus* phages are very host specific. Sometimes phage stocks produced only a dim inhibition area on alternative hosts (presented as light gray coloring in **Figure 2**). This suggests that something, but not necessarily the phage in the prepared stocks was restricting the growth of the bacterium. Furthermore, phages isolated for any particular host often had similar infection patterns. This suggests that additional isolation attempts using the same isolation source for enrichment may not be the best choice for improving the host-range of the cocktail.

DISCUSSION

Due to the enormous variety of bacteriophages in environmental reservoirs, on-demand isolation of novel phage-antibacterials is a potential way to generate a personalized medicine for treating bacterial infections that are resistant to conventional drugs. In this study, we evaluated the feasibility of isolating phages for such therapeutic cocktails.

The efforts required to find phages differs substantially between bacterial species. Phages can be readily discovered for *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *Salmonella* species. Although virus production was neither optimized nor standardized in this study, phages of these hosts also readily generated high-density virus stocks (**Figure 1**). In contrast, we found it very challenging to isolate phages against *Staphylococcus* strains despite of several attempts that were conducted at different times of year and from multiple sources (sewage, river, hospital water lock, and livestock farm soil samples). It was also more laborious to isolate phages for *E. faecium* and *faecalis* and *A. baumannii,* although it must be noted that we had only handful of these strains and we performed only few isolation attempts for them. Nevertheless, based on the results, the on-demand discovery of phages appears to be feasible for some but not all bacteria. This highlights the importance of premade wide-host range cocktails or the existence of other antimicrobial solutions against species such as *S. aureus* (such as the one developed by Kelly et al., 2011). Also, teixobactin, the first new potential antibiotic to be discovered in 30 years is very effective against bacteria lacking the outer membrane (such as *S. aureus* and *Enterococcus*; Ling et al., 2015). Yet, gram-negative pathogens with the impermeable outer membrane (e.g., *E. coli*, *Salmonella*, *K. pneumonia,* and *P. aeruginosa*) are inherently resistant to antibiotics like teixobactin, but contrastingly appear to be suitable targets for obtaining a cocktail from environmental reservoir (sewage) as needed. Also, better preservability and wider host-range of these phages supports the on-demand isolation approach. While conventional tools for antibiotic development may still remain relevant, in the face of worsening world-wide antibiotic resistance crisis we should be actively exploring these promising alternatives in order to retain the upper hand against all pathogens.

Generalization of the obtained results must be done while acknowledging the potential sources of error. First, while we collected our sewage samples at different times (over the timespan of almost 2 years), only a single wastewater management plant was used. Although the biological material in these plants changes constantly, the phage populations may still be substantially different in different plants, thus possibly skewing the chances for finding phages against certain species. Moreover, the host ranges of some phages appear identical, suggesting that the hosts themselves may be genetically very close to one another. Second, albeit we performed several hundred isolation attempts, just a few isolations were performed for any particular strain and thus the achieved probabilities should be treated as a case study rather than an exhaustive evaluation. Third, we did not perform an in-detail characterization for the isolated phages. Such characterization, at least to some extent, will be necessary during actual therapy practices (Skurnik et al., 2007; Merabishvili et al., 2009; Keen, 2012), as bacteriophages are known to carry undesirable genes coding for toxins and antibiotic resistances (Loc-Carrillo and Abedon, 2011). However, separating lytic phages from temperate phages (possibly when accompanied with genome sequencing and analysis) should be enough and feasible for the rapid assessment of safety (Chan et al., 2013). Also, phage stocks have to be purified from (host-bacterium generated) endotoxins before therapeutic use (Keen, 2012). These steps were not performed or their effects on phages evaluated in this study.

CONCLUSION

The success of on-demand isolation of phages appears to be critically dependent on the bacterial host. Promisingly, against pathogens for which conventional antibiotics are becoming the least useful, such as ESBL *E. coli* and *K. pneumoniae*, personalized phage therapy could be considered as a potential alternative.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01271

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SLPW: A Virulent Bacteriophage Targeting Methicillin-Resistant *Staphylococcus aureus In vitro* and *In vivo*

Zhaofei Wang, Panpan Zheng, Wenhui Ji, Qiang Fu, Hengan Wang, Yaxian Yan * and Jianhe Sun *

Shanghai Key Laboratory of Veterinary Biotechnology, Key Laboratory of Urban Agriculture (South), Ministry of Agriculture, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China

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***Correspondence:**
Yaxian Yan
yanyaxian@sjtu.edu.cn;
Jianhe Sun
sunjhe@sjtu.edu.cn

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Staphylococcus aureus (*S. aureus*) is a Gram-positive pathogen causing a variety of infections in humans and animals. Extensive use of antibiotics has led to the emergence of methicillin-resistant *S. aureus* (MRSA). As an alternative antibacterial agent against drug-resistant *S. aureus*, a lytic phage, designated SLPW, was isolated from fecal sewage in a pig farm. The SLPW was morphologically classified under *Podoviridae* and contains a double-stranded DNA genome. The genome of SLPW was 17,861 bp (29.35% G+C) containing 20 open reading frames and lacked regions encoding lysogeny-related integrase gene and *cI* repressor gene. Phage SLPW showed a broad host range and high efficiency of plating against various types of *S. aureus*. One-step growth curve showed a short latency period (10 min) and a long lytic period (120 min). Phage SLPW remained stable under a wide range of temperatures or pH and was almost unaffected in chloroform or ultraviolet light. Further, it efficiently lysed MRSA strains *in vitro* and *in vivo*. Intraperitoneal phage administration at 1 h post-infection cured the mice and reduced the bacterial expression of inflammatory cytokines in mice. Specifically, the phage SLPW displayed a wide antibacterial spectrum. It was therapeutically effective against intra-abdominal infection in mice harboring different multilocus sequence typing (MLST) types of *S. aureus* strains. Therefore, phage SLPW is a potential therapeutic agent against MRSA infections.

Keywords: *Staphylococcus aureus*, MRSA, phage SLPW, infection, therapy

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is the most virulent pathogen causing various diseases, including skin abscesses, pneumonia, endocarditis, and osteomyelitis, in humans and animals (Lowy, 1998; Plata et al., 2009). The two major sources of infection include community and hospital (Engemann et al., 2003). The bacterial strains are resistant to many antibiotics, and especially to methicillin and vancomycin. The emergence and prevalence of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) underscores the need for development of effective therapeutic alternatives (Sasidharan et al., 2011; Gardete and Tomasz, 2014).

In the last 15 years, there has been a marked increase in the number of identified *Staphylococcus* phages, with tremendous progress in therapeutic interventions targeting *Staphylococcus*, especially

S. aureus (Hsieh et al., 2011). Phages are the most common organisms on the planet and represent great diversity in host range. *S. aureus* phages target pathogens in diseases, such as bacteremia, eye infections, and *S. aureus*-associated lung infections (Wills et al., 2005; Kazmierczak et al., 2015). Compared with traditional antibiotics, bacteriophages are cost-effective without serious side effects, and are virulent especially against drug-resistant bacteria (Borysowski et al., 2011; Kazmierczak et al., 2015). Further, phages generally recognize specific receptors on bacterial cell membrane, without affecting human, or animal cells. Therefore, the side effects in eukaryotic hosts are minimal (Sulakvelidze et al., 2001). Studies involving *S. aureus* phages show effective and comprehensive antimicrobial activity *in vitro* and *in vivo* (Capparelli et al., 2007; Gutierrez et al., 2015).

In this study, we isolated a lytic phage, SLPW, from fecal sewage in a pig farm. We report the wide host range, adequate stability and strong bacteriolytic activity of this phage. Specifically, the phage SLPW was safe and effective against MRSA infection in mice.

MATERIALS AND METHODS

Ethics Statement

Animal experiments were carried out according to the animal welfare standards approved by the Ethical Committee for Animal Experiments of Shanghai Jiao Tong University, China. All animal experiments complied with the guidelines of the Animal Welfare Council of China.

Bacterial Strains and Culture Conditions

In this study, 38 *S. aureus* strains (18 MRSA strains, 7 clinically isolated pathogenic strains and 13 strains isolated from milk samples of dairy cows with mastitis) and 8 other strains (*Staphylococcus epidermidis* ATCC12228, *Bacillus subtilis* YS, *S. zooepidemicus* ATCC35246, 4 *Streptococcus suis* and *Escherichia coli* MC1061) were used (**Table 1**). Two reference strains of *S. aureus* ATCC 25923 and ATCC 29213 from the American Type Culture Collection (ATCC) were also used. All the strains were grown in Todd–Hewitt broth (THB) and brain heart infusion (BHI) or agar medium supplemented with 2% (vol/vol) fetal bovine serum (Gibco, Invitrogen Corp., Carlsbad, CA) at 37°C.

Phage Isolation, Purification, and Host Range Determination

The method of Matsushiro et al. was adopted for the isolation of *S. aureus* phages with some modifications (Matsushiro and Okubo, 1972). Seventy-four samples, including 44 dust swabs and 30 fecal samples were suspended in SM buffer [NaCl 5.8 g/L, $MgSO_4 \cdot 7H_2O$ 2 g/L, 1 M Tris·HCl (pH7.5) 50 ml/L, and 2% gelatin 5 ml/L] and centrifuged at 5000 × *g* for 20 min at 4°C. The supernatants were filtered through 0.22-μm pore membranes and evaluated for the presence of lytic phages using different *S. aureus* isolates on BHI plates. After overnight incubation, bacterial plaque formation suggested the presence of lytic phage, which was purified after three rounds of single-plaque isolation.

For purification, a single-phage plaque was precipitated in the presence of 10% (wt/vol) polyethylene glycol (PEG) 8000 and 1

TABLE 1 | SLPW strains and lytic activity

Species of strain	No. of strain	MLST type	EOP[a]	Source[b]
Staphylococcus aureus (22)	ATCC25923	ST 243	1	III
	ATCC29213	ST 5	1.37×10^{-5}	III
	S1	ST 398	4.24×10^{-6}	I
	S2	ST 239	6.12×10^{-1}	I
	S3	ST 239	—	I
	S4	ST 239	2.13×10^{-1}	I
	S5	ST 398	6.33×10^{-1}	I
	S6	ST 9	—	I
	S7	ST 239	8.22×10^{-5}	I
	SH-5	ST 9	2.32×10^{-1}	II
	SH-6	ST 9	5.44×10^{-7}	II
	SH-7	ST 9	7.21×10^{-6}	II
	SH-8	ST 9	8.14×10^{-1}	II
	SH-9	ST 9	2.02×10^{-1}	II
	SH-10	ST 9	4.37×10^{-1}	II
	SH-11	ST 9	2.62×10^{-1}	II
	SH-12	ST 9	4.23×10^{-1}	II
	SH-13	ST 9	4.22×10^{-5}	II
	SH-14	ST 9	9.17×10^{-6}	II
	SH-15	ST 9	1.98×10^{-1}	II
	SH-16	ST 9	2.82×10^{-6}	II
Methicillin-resistant *Staphylococcus aureus* (18)	SH-17	ST 9	1.81×10^{-1}	II
	MS3	ST 9	1.62	IV
	MS5	ST 5	7.52×10^{-1}	IV
	MS6	ST 5	2.88×10^{-1}	IV
	MS7	ST 5	7.23×10^{-1}	IV
	MS8	ST 9	8.22×10^{-1}	IV
	MS9	ST 9	8.21×10^{-1}	IV
	MS10	ST 239	2.82×10^{-6}	IV
	MS11	ST 9	4.33×10^{-1}	IV
	MS13	ST 9	8.22×10^{-1}	IV
	MS15	ST 5	8.17×10^{-1}	IV
	MS16	ST 9	9.23×10^{-1}	IV
	MS17	ST 398	4.33×10^{-5}	IV
	MS18	ST 9	3.22×10^{-1}	IV
	MS19	ST 9	2.98×10^{-1}	IV
	MS20	ST 398	1.23×10^{-1}	IV
	MS21	ST 398	1.67×10^{-1}	IV
	MS22	ST 398	—	IV
	MS23	ST 9	—	IV
Staphylococcus epidermidis	ATCC12228	—[c]	—	IV
Bacillus subtilis	YS	—	—	IV
Streptococcus suis	SS1	—	—	IV
	SS2	—	—	IV
	SS7	—	—	IV

(Continued)

TABLE 1 | Continued

Species of strain	No. of strain	MLST type	EOP[a]	Source[b]
	SS9	—	—	IV
Streptococcus zooepidemicus	ATCC35246	—	—	III
Escherichia coli	MC1061	—	—	IV

[a] *EOP, efficiency of plating (EOP = phage titer on test bacterium / phage titer on host bacterium Staphylococcus aureus ATCC25923). Assays were conducted at least three times. The data shown represent means derived from three independent experiments.*
[b] *I, clinically-isolated pathogenic strains; II, isolated from milk samples of dairy cows with mastitis; III, purchased from American Type Culture Collection; IV, stored in our lab.*
[c] *—, no plaque on target bacterium.*

M NaCl at 4°C for at least 1 h. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min at 4°C and suspended in SM buffer. After the addition of 0.5 g/mL CsCl, the mixture was layered on top of CsCl step gradients (densities of 1.15, 1.45, 1.50, and 1.70 g/mL) in Ultra-Clear centrifugation tubes and centrifuged at 28,000 × *g* for 2 h at 4°C, and dialyzed in sodium chloride–magnesium sulfate buffer [100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, and 50 mM Tris·HCl (pH 7.5)]. Phages were stored at 4°C for further experiments.

The host range of the phage was defined by the double-layered agar method described by Adams (1959). The SLPW phage was inoculated with all the 47 strains listed in **Table 1** and then monitored for plaque formation.

Transmission Electron Microscopy (TEM) of Phage Particles

The purified phage sample was loaded onto a copper grid for 7 min followed by negative staining with 2% (vol/vol) uranyl acetate (pH 6.7) and drying. The phage morphology was observed using a FEI TEM Tecnai G2 Spirit Biotwin (FEI, Hillsboro, US) at an accelerating voltage of 80 kV.

Restriction Enzyme Digestion of Phage Genomic DNA

Purified phage genomic DNA was prepared as described previously by Son et al. (2010). For the identification of nucleic acid type, purified phage genomic DNA was subjected to nuclease treatment using D*Nase* I (20 U/μL), R*Nase* A (5 U/μL), and Mung bean nuclease (20 U/μL) at 37°C for 1 h. Restriction site analysis of the phage was conducted by digesting purified phage genomic DNA with 10 U *Xho* I, *EcoR* I, *Hind* III, *Ned* I, and *Not* I for 1 h at 37°C. Products of digested phage nucleic acid were separated by 0.8% (wt/vol) agarose gel electrophoresis.

Genome Sequencing and Annotation

Shotgun sequencing was used for Phage SLPW whole genome analysis. Sequence alignments were carried out using the Accelrys DS Gene software package of Accelrys Inc. (USA). Putative open reading frames were suggested using the algorithms of the software packages Accelrys Gene v2.5 (Accelrys Inc.) and ORF Finder (NCBI). Identity values were calculated using different BLAST algorithms (http://www.ncbi.nlm.nih.gov/BLAST/) at the NCBI homepage. The sequence of phage SLPW has been submitted to NCBI (GenBank accession number: KU992911).

Assay of Optimal Multiplicity of Infection (MOI)

Overnight cultures of *S. aureus* ATCC 25923 strain were diluted 1:100 in fresh BHI and incubated at 37°C with shaking until early logarithmic growth phase (optical density at 600 nm, 0.4–0.6), diluted 1:10, and mixed with phages at different MOIs. After 3.5 h incubation at 37°C, the mixture was centrifuged at 5000 × *g* for 20 min at 4°C and the supernatants were filtered through 0.22-μmporesize membranes. The phage titer in the supernatant was immediately determined using a double-layer agar plate method. This assay was performed at least in triplicate.

One-Step Growth

For determination of one-step growth of phage SLPW, we used *S. aureus* ATCC 25923 as the host strain because it displayed the largest clearance zone in a spot test and was lysed rapidly to yield a clear lysate in liquid culture. One-step growth experiments were performed using a modified method described previously (Pajunen et al., 2000). Briefly, SLPW phage was added at a MOI of 0.1 to the cells of *S. aureus* and allowed to adsorb for 15 min at 37°C. The mixture was then centrifuged at 10,000 × *g* for 1 min. After the supernatants were removed, the pellets containing the phage-infected bacterial cells were suspended in fresh BHI and incubated with shaking at 180 rpm and 37°C. Partial samples were obtained at 10 min intervals and the titrations from the aliquots were immediately determined using the double-layer agar plate method. This assay was performed at least in triplicate.

Phage Stability

Phage stability was determined at different temperatures (25, 37, 45, 50, 55, 60, 65, and 70°C), using an aliquot of phage SLPW obtained after 1 h. The titers of the phage lysate were assayed using a double-layer agar plate method. The phage stability at different pH-values was tested by determining the titers after dilution of the phage lysates (1:100) in SM buffer and stored at 37°C for 3 h. To analyze the chemical stability, the phage SLPW was treated with chloroform (5, 25, 50, or 75%, vol/vol) for 6, 12, 18, and 24 h at 4°C. In addition, the phage SLPW was also exposed to ultraviolet light treatment for 10, 20, 30, 40, 50, and 60 min, and titrated immediately using a double-layer agar plate method.

Efficiency of Plating

Phage SLPW was screened against *S. aureus* strains using the efficiency of plating method (EOP = phage titer on test bacterium/phage titer on host bacterium) to determine the effectiveness against a variety of target bacteria. Ten-fold serial dilutions of phage suspensions (100 μL) were mixed with 100 μL of the target or host bacterium (grown overnight at 37°C) and incubated for 5 min at room temperature (25°C) and plated as double layers on THB (Viscardi et al., 2008).

Phage Bacteriolytic Activity *In vitro*

Overnight cultures of *S. aureus* culture were diluted 1:100 in fresh THB liquid medium incubated at 37°C with shaking at 180 rpm until an early-exponential host bacterial culture (optical density at 600 nm, 0.4–0.6) was reached. Phage SLPW was added at MOI of 0.01, 1, and 100, and an identical *S. aureus* culture with the same volume phage diluent was used as the control. The mixture was then grown at 37°C with shaking at 180 rpm. The phage bacteriolytic activity was assessed by monitoring the cell absorbance of the culture solution (OD_{600}) at 30-min intervals for up to 4 h, and this assay was performed in triplicate.

Phage Protection Studies

Female BALB/c mice (6 weeks of age) were purchased from the Experimental Animal Center, Shanghai Jiao Tong University. Overnight cultures of *S. aureus* were diluted 1:100 in fresh THB liquid medium incubated at 37°C with shaking at 180 rpm to an early-exponential host bacterial culture (optical density at 600 nm, 0.4–0.6). Cells were pelleted and washed twice with phosphate-buffered saline (PBS). The mice were infected with a dose of 1×10^9 CFU in 0.2 mL of the *S. aureus* strain. The bacterial cells were injected unilaterally into the abdominal cavities of mice, and 0.2 mL of the purified phage samples (1×10^9 PFU) were injected into the other side immediately, at 60 and 120 min after a bacterial challenge. The controls included uninfected mice administered with 0.2 mL of phage in SM buffer. The mouse survival rate was recorded daily for 7 days. The CFU or PFU organ burden in spleen, lung, and blood was determined by sacrificing groups of six mice at 6, 12, and 24 h after phage SPLW administration. Each sample was homogenized in 1 mL PBS and serially diluted in PBS. CFU were evaluated by plating each dilution on THB agar plates. The PFU were evaluated by plating each dilution on the double-layer agar plate.

Cytokine Assays

To evaluate the antimicrobial effects of phage, 0.2 mL of phage in SM buffer (1×10^9 PFU) was administered intraperitoneally at 1 h after infection with *S. aureus* (1×10^9 CFU). SM buffer alone was administered to uninfected mice serving as control groups. Spleen tissues were removed from mice 6, 12, and 24 h after injection with phage SLPW. Tissues were homogenized in 1 mL of lysis buffer (Qiagen, West Sussex, UK), followed by centrifugation at 2000 × *g* for 10 min. The supernatants were sterilized with a millipore filter (0.45-μm pore size).

Total RNA was isolated from the supernatants using an AllPrep RNA microkit (Qiagen). The cDNA synthesis was performed using the PrimeScript RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The mRNA levels were measured using two-step relative qRT-PCR. The β-actin housekeeping gene was amplified as an internal control. The sequences of the primers for tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and β-actin are listed in **Table 2**. Gene expression was normalized to the expression of the housekeeping gene β-actin. Real-time PCR was performed using a SYBR Premix Ex Taq kit (TaKaRa) and CFX Connect^{TM} RT-PCR system (BIO-RAD, Hercules, USA).

TABLE 2 | Primers used for qRT-PCR

Primer	Sequence (5′–3′)
IL-1β-F	TCCAGGATGAGGACATGAGCAC
IL-1β-R	GAACGTCACACACCAGCAGGTTA
IL-6-F	CCACTTCACAAGTCGGAGGCTTA
IL-6-R	GCAAGTGCATCATCGTTGTTCATAC
TNF-α-F	AAGCCTGTAGCCCACGTCGTA
TNF-α-R	GGCACCACTAGTTGGTTGTCTTTG
β-actin-F	TGACAGGATGCAGAAGGAGA
β-actin-R	GCTGGAAGGTGGACAGTGAG

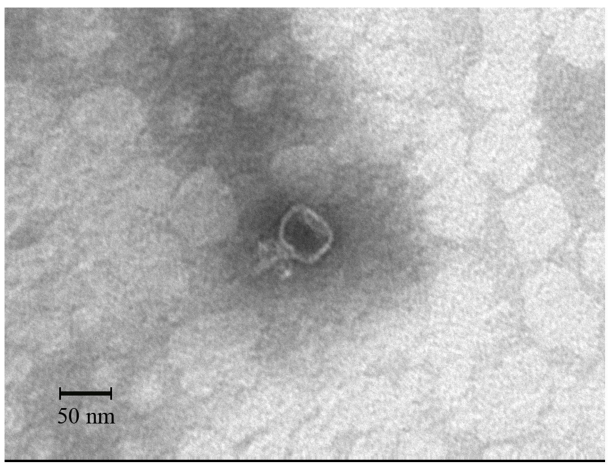

FIGURE 1 | Transmission electron microscopy of negatively-stained phage SLPW.

The comparative cycle threshold ($2^{-\Delta\Delta CT}$) method was used to analyze the mRNA levels.

Statistical Analyses

Experimental data points were plotted using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). Data were expressed as mean values ± standard errors of the means (SEM). The phage protection analyses were performed using the non-parametric Mann-Whitney U-test. A $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Phage Isolation and Host Range Determination

In this study, we isolated a lytic *S. aureus* phage designated as SLPW, from fecal sewage in a pig farm of Shanghai (China) in 2013. Using *S. aureus* ATCC25923 as the host strain, the phage plaques measuring 1–2 mm in diameter were obtained. The phage SLPW had a strong ability to produce plaques on *S. aureus* strains. Among the 40 *S. aureus* strains, 36 (90%) isolates were lysed by SLPW (**Table 1**). Furthermore, the SLPW phage showed strong lytic activity against the majority of MRSA

strains (16 of 18 strains), suggesting a potential therapeutic role in MRSA infection. However, no plaque production was observed in *Staphylococcus epidermidis*, *Bacillus subtilis*, *S. zooepidemicus*, *Streptococcus suis,* and *E. coli* strains investigated (**Table 1**).

The morphology of the isolated phage SLPW was determined. Electron microscopy showed that the SLPW particle had an isometric head of 49.5 ± 1.5 nm and a short, non-contractile tail measuring 19.5 ± 1.5 nm long (**Figure 1**). Thus, it was morphologically similar to phages of the family *Podoviridae* according to the classification of International Committee on Taxonomy of Viruses (ICTV; Adams et al., 2014).

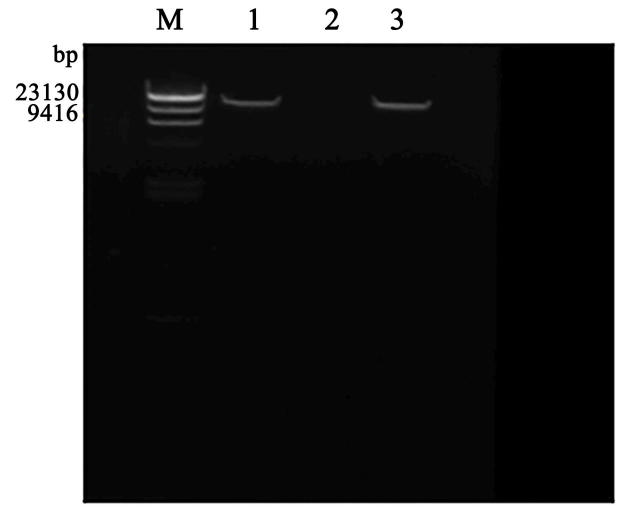

FIGURE 2 | Agarose gel electrophoresis of phage SLPW genome digested with nuclease. Lane M: λ-*Hind* III digest DNA Marker, Lane 1–3: phage SLPW genome digested with R*Nase*A, D*Nase* I, and Mung Bean Nuclease, respectively.

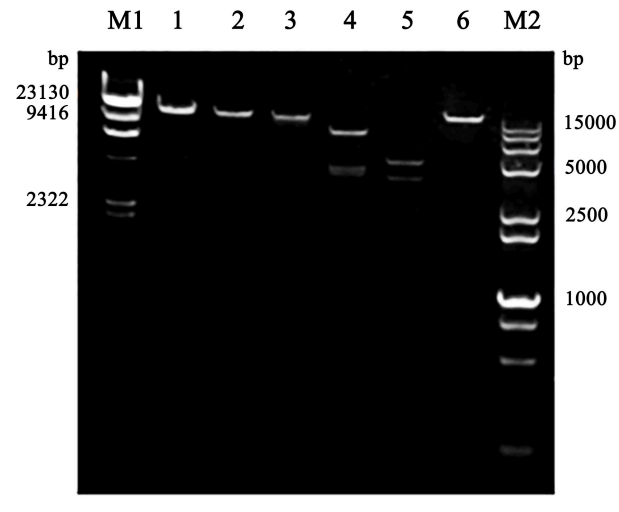

FIGURE 3 | Restriction digestion patterns of phage SLPW DNA. Lane M1: λ-*Hind* III digested DNA Marker, Lane 1: phage SLPW genome, Line 2–6: phage SLPW genome digested with *Xho* I, *EcoR* I, *Hind* III, *Ned* I, and *Not* I, respectively, Line M2: DL 15000+2000 marker.

Phage Nucleic Acid Type and Genome Description

The purified phage genomic DNA was subjected to digestion by different nucleases. The results showed that the genome of phage SLPW was completely digested by D*Nase* I but not by R*Nase* A or Mung bean nuclease (**Figure 2**), suggesting that phage SLPW was a double-stranded DNA. Purified phage SLPW genomic DNA could be digested with several restriction endonucleases including *Xho* I, *EcoR* I, *Hind* III, and *Ned* I (**Figure 3**).

The complete nucleotide sequence of phage SLPW was determined. The SLPW genome comprises 17,861 bp with an average G+C content of 29.35%, which is similar to that of the lytic *Staphylococcus* phages S13', PSa3, and 66 (Table S1).

TABLE 3 | Optimal multiplicity of infection (MOI) of phage SLPW.

CFU of *S. aureus* ATCC25923 strain	PFU of phage SLPW	MOI	Phage SLPW titers (PFU/mL)
10^6	10^8	100	3.32×10^9
10^7	10^8	10	2.71×10^9
10^8	10^8	1	3.12×10^{10}
10^8	10^7	0.1	4.87×10^{10}
10^8	10^6	0.01	2.92×10^8
10^8	10^5	0.001	4.41×10^7
10^8	10^4	0.0001	3.37×10^6

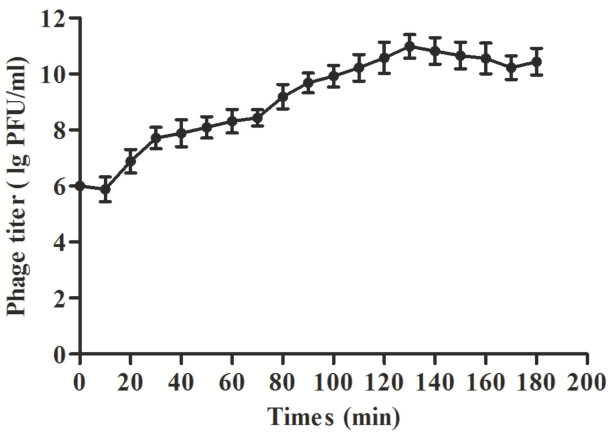

FIGURE 4 | One-step growth curve of phage SLPW in *S. aureus*. Phage SLPW was co-incubated with *S. aureus* ATCC25923 strain cultured at an MOI of 0.1 for 15 min at 37°C. The mixture was centrifuged to remove non-absorbed phage. The re-suspended pellets were incubated at 37°C and sampled at 10 min intervals over a period of 3 h. Phage titer was measured. Results are shown as means ± SEM from triplicate experiments. The latent period was 10 min: interval between the absorption and the beginning of the initial burst. The burst size was estimated at 95.3 PFU per infected cell, which was the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells.

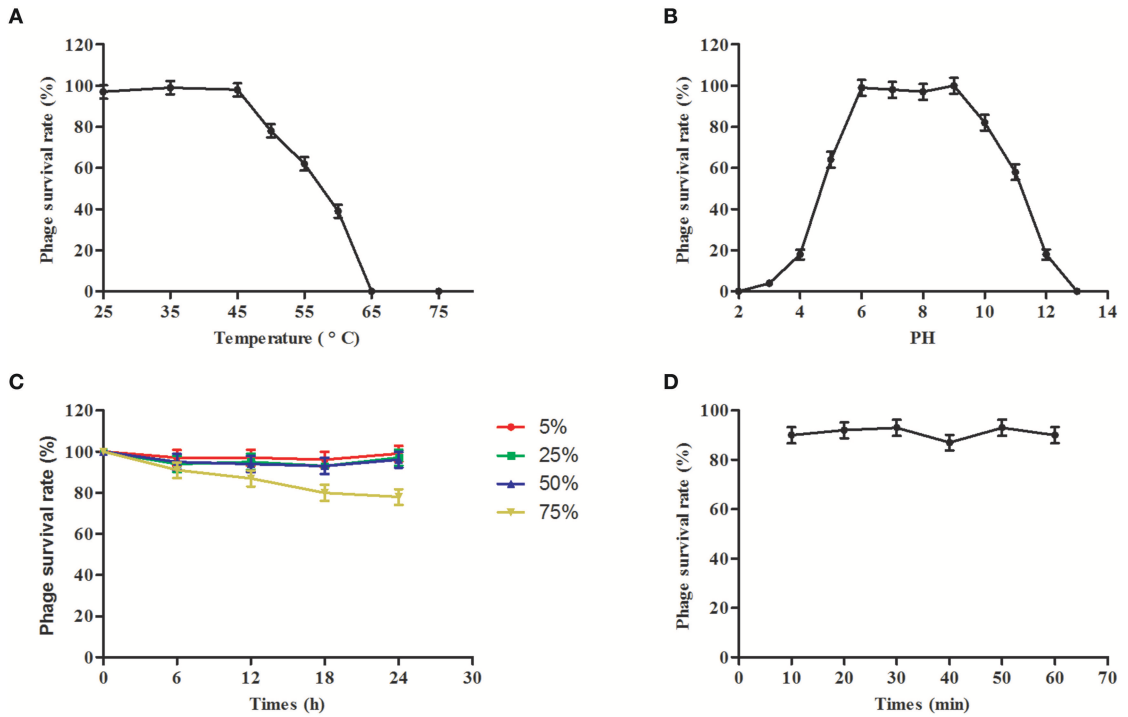

FIGURE 5 | Stability tests of phage SLPW. (A) Thermostability: Phage SLPW was incubated at various temperatures as indicated. Samples were collected after 1 h; **(B)** pH stability: Phage SLPW was incubated under different pH conditions for 3 h; **(C)** Chloroform stability: Phage SLPW was treated with chloroform (5, 25, 50, or 75%, vol/vol) for 6, 12, 18, and 24 h; **(D)** Ultraviolet light stability: Phage SLPW was exposed to UV light for 10, 20, 30, 40, 50, and 60 min. The overall results were expressed as survival rates, and were titrated immediately using double-layer agar plate method. Results are shown as means ± SEM from triplicate experiments.

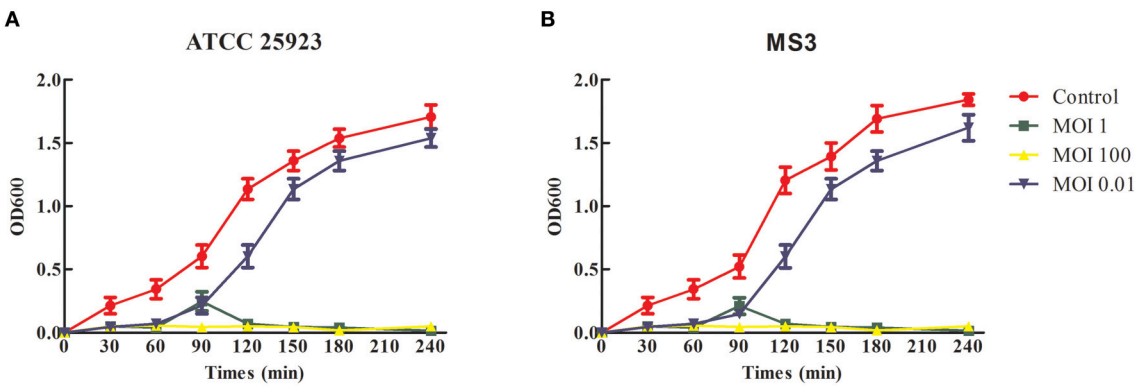

FIGURE 6 | Bacteriolytic activity of SLPW against *S. aureus in vitro*. Early exponential cultures of *S. aureus* **(A)** ATCC25923 and **(B)** MS3 strains were co-cultured with SLPW phage at MOIs of 0.01, 1, and 100, respectively. *S. aureus* cultured with a similar volume of phage diluent was used as a control. Results are shown as means ± SEM from triplicate experiments.

As shown in Table S2, 20 open reading frames (ORFs) were defined as potential genes of SLPW. Genes involved in packaging, head, tail, lysis, and DNA replication showed high homology with other phages listed in Figure S1 and Table S2 (Kwan et al., 2005). However, a few unknown proteins of SLPW including Gp4, Gp6, and Gp20 showed a lower degree of similarity than those of other *Podoviridae Staphylococcus* phages (Table S2), which may lead to functional discrepancy. The genes of SLPW encoding lysin (Gp14) and holin (Gp10) showed high similarity with *Staphylococcus* lytic phages listed in Table S2, which also showed a broad host range and strong lytic ability against *S. aureus* (Kraushaar et al., 2013). Comparison of the genome structure with *Staphylococcus* prophages suggested that SLPW was a lytic phage, which lacked specific integration-related and *cI* repressor genes, devoid of lysogenic characteristics (Kwan et al., 2005; Hoshiba et al., 2010; Biswas et al., 2014).

Determination of Optimal Multiplicity of Infection (MOI) and One-Step Growth Curve

The results showed that the optimal MOI of phage SLPW was 0.1, which was the highest titer attained by the phage lysates (4.87×10^{10} PFU/mL; **Table 3**). Based on the optimal MOI, we established a one-step growth curve. Short latency period (10 min) and large burst size (estimated at 95.3 PFU per infected cell; **Figure 4**) suggest lytic nature of the SLPW phage and a higher lytic activity than the previously published lytic *S. aureus* phages (Han et al., 2013; Li and Zhang, 2014).

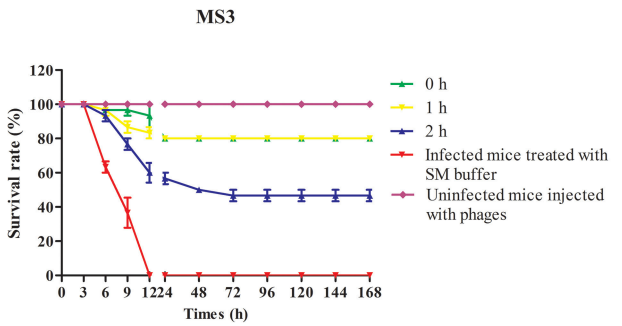

FIGURE 7 | Survival curves of mice infected with *S. aureus* MS3 strain and treated with phage SLPW. Phage (MOIs of 1) was administered intraperitoneally into mice at 0, 1, or 2 h post-infection and the survival rates were recorded. Data shown are representative of three independent experiments using 10 mice per group, and displayed as mean ± SEM.

Phage Stability

The potential clinical role of phage SLPW was evaluated by determining their physical and chemical stabilities. The thermal stability of the phages was investigated at different temperatures. We found that the activity of phage SLPW was stable at temperatures up to 45°C. Higher temperatures resulted in progressive inactivation. Phage SLPW was completely inactivated when heated to 65°C (**Figure 5A**). The pH stability was studied in SM buffers at a pH range of 2–12. Phage SLPW showed a relatively high survival rate (more than 80%) at a pH ranging from 6 to 10. Beyond these values, the activity decreased dramatically (**Figure 5B**). Further, the viability of phage SLPW was almost unaffected in the presence of 5, 25, 50, and 75% chloroform as shown in **Figure 5C**. Ultraviolet irradiation assay showed that about 90% of phage SLPW survived UV light (30 w, 30 cm wave-length) treatment ranging from 10 to 60 min (**Figure 5D**).

Studies suggested a probable relationship between phage structure and survival under adverse environmental conditions (Lasobras et al., 1997). Ackermann et al indicated that tailed phages remain comparatively steady in adverse conditions (Ackermann et al., 2004). Under harsh conditions, such as strong ultraviolet light, and large temperature fluctuations, phages belonging to *Myoviridae* protect themselves from extremely dry environment via intercellular location in pseudo-lysogens

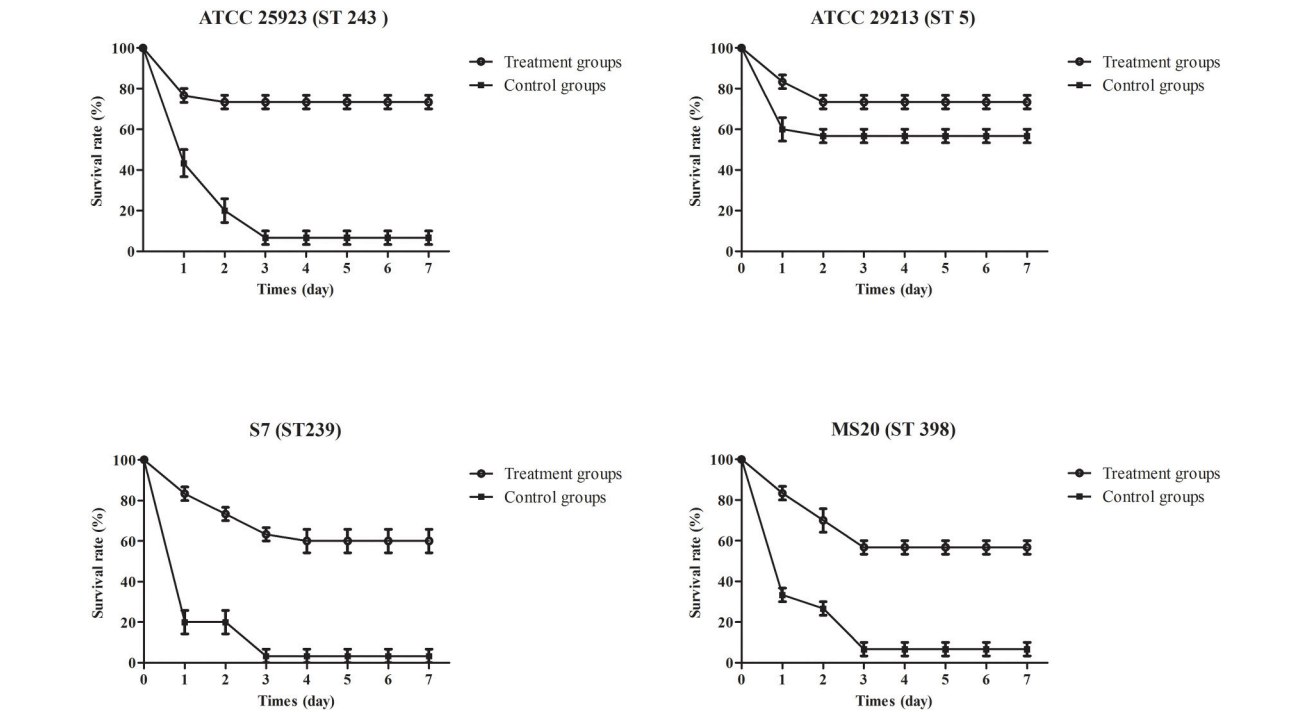

FIGURE 8 | Survival curves of mice infected with different *S. aureus* strains and treated with phage SLPW. Phage (MOIs of 1) was injected intraperitoneally into mice at 1 h post-infection and the survival rates were recorded. Infected mice treated with SM buffer served as control. Data shown are representative of three independent experiments using 10 mice per group, and displayed as mean ± SEM.

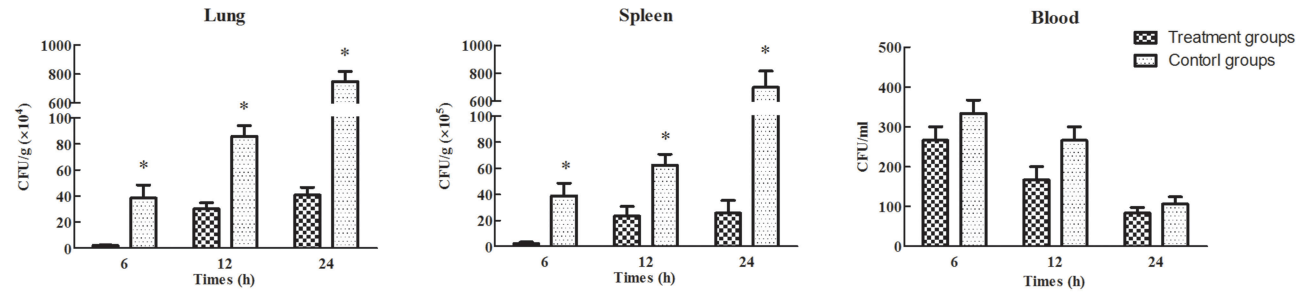

FIGURE 9 | *S. aureus* concentrations in the blood and organs of mice treated with phage SLPW at 1 h post-infection. Six mice were sacrificed from each mouse group at different time points. SM buffer was administered to infected mice, which served as control. Results are shown as means ± SEM from triplicate experiments. The Mann-Whitney U-test was used to compare the phage concentration data. Significant differences ($P < 0.01$) are indicated by asterisks.

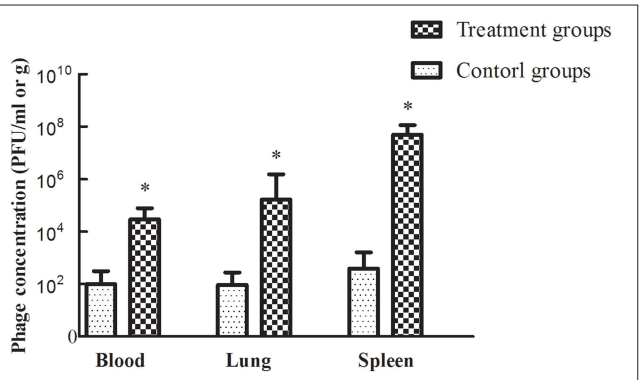

FIGURE 10 | Figure 10 Phage SLPW concentrations in the blood and organs of mice treated with phage SLPW at 1 h post-infection. Six mice were sacrificed from each group at 24 h after the experiment. Phage was injected into uninfected mice, which served as controls. Results are shown as means ± SEM from triplicate experiments. The Mann-Whitney U-test was used to compare the phage concentration data. Significant differences ($P < 0.01$) are indicated by asterisks.

or biofilms created by bacterial hosts (Jonczyk et al., 2011). Phages from *Podoviridae* family may be extremely resistant to dry environment and survive large temperature fluctuations (Prigent et al., 2005). Our studies suggest that SLPW, which belongs to *Podoviridae*, showed a broad range of thermal and pH stability and strong resistance to chloroform and ultraviolet light treatment. Based on the above studies, tailed phages generally show great ability to adapt to adverse conditions, contributing to the development of phagotherapy.

Bacteriolytic Activity *In vitro*

The phage SLPW bacteriolytic activity was tested in an early-exponential phase culture of *S. aureus* ATCC25923 and MS3 strains. The growth of these strains steadily declined at an MOI 1 and was completely inhibited at MOI 100 directly after phage administration (**Figure 6**). However, when the culture was administered using phage SLPW at MOI 0.01, the absorbance (OD600) continued to increase during the incubation (**Figure 6**). The results suggested that SLPW was highly effective against *S. aureus in vitro* and an MOI 1 was used for therapeutic study *in vivo*.

Phage Therapeutic Study

Although studies indicate successful outcomes with topical phage treatment of human and animal infections involving *S. aureus*, few studies focused on the treatment of acute and lethal infection (Chhibber et al., 2013; Pincus et al., 2015). To investigate the virulence of MRSA strain MS3 in BALB/c mice, the mortality of mice was recorded daily, and followed over a period of 7 days after infection. Injection with 1×10^9 and 1×10^7 bacterial cells resulted in 100 and 0% death, respectively (data not shown). These doses were therefore used in subsequent experiments.

To evaluate the therapeutic potential of phage SLPW *in vivo*, assays were performed using BALB/c mice after infection with high concentrations of *S. aureus* (1×10^9 CFU/mouse). The results showed that mice treated with phage immediately (0 h) or at 1 h post-infection showed significantly higher survival rates than the control groups (infected mice treated with SM buffer) after 7 days. The survival rates following phage therapy at 0, 1, and 2 h were about 80, 80, and 50% after 7 days, respectively (**Figure 7**). Recent studies have shown that immediate phage treatment provided better protection than delayed administration against bacterial infection in mice (Watanabe et al., 2007; Hsieh et al., 2011). However, our study indicated that the survival rates between immediate and delayed therapy (1 h post-infection) by phage SLPW were similar (**Figure 7**). This finding confirmed that phage SLPW showed higher efficiency and sensitivity against *S. aureus in vivo*. In addition, phage SLPW exhibited satisfactory therapeutic effect against different sequence types of *S. aureus* following multilocus sequence typing (MLST; **Figure 8**).

Treatment efficacy was evaluated by examining bacterial colonization in the organs and blood of mice after phage therapy (1×10^7 PFU/mouse) following 1 h of infection with a non-lethal dosage of *S. aureus* (1×10^7 CFU/mouse). Mice infected with *S. aureus* strains showed relatively high pathogen density in organs (Takemura-Uchiyama et al., 2014; Li et al., 2015). However, the mice treated with SLPW showed significantly lower *S. aureus* levels in the spleen and lung than the control groups at every time point (**Figure 9**) suggesting that SLPW was therapeutically effective against systemic infection caused by *S. aureus*. However, the *S. aureus* concentrations in the blood of the phage-administered groups were slightly lower than in the control groups (**Figure 9**). The results also showed that

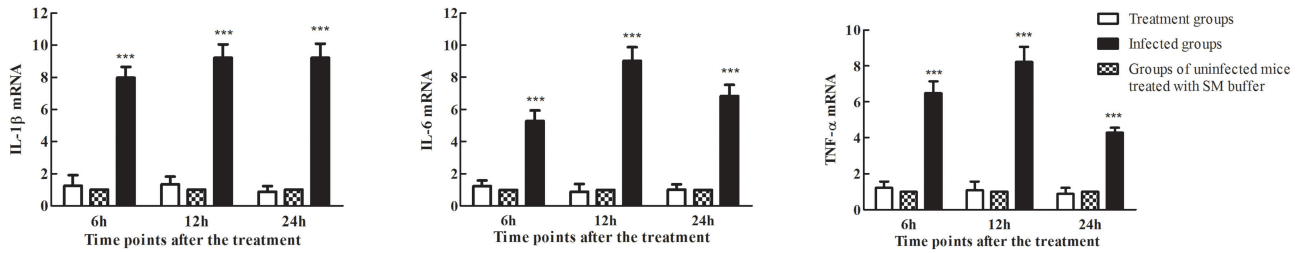

FIGURE 11 | Levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the spleens of mice treated with phage SLPW at 1 h after infection. Six mice were sacrificed from each mouse group at different time points. SM buffer was administered to uninfected control mice. The values of pro-inflammatory cytokines in the control groups were normalized to 1.0. Levels of IL-1β, IL-6, and TNF-α mRNA were normalized to β-actin mRNA levels and were expressed as n-fold increases with respect to the control. Results are shown as means ± SEM from triplicate experiments. The Mann-Whitney U-test was used to compare the cytokine levels between infected groups and treatment groups. Significant differences ($P < 0.001$) are shown by asterisks.

phage titers in the blood and organs at 24 h after therapy were significantly increased by nearly four orders of magnitude in the spleen and two orders of magnitude in the blood and lung compared with those in the uninfected control groups (**Figure 10**). Based on these results, we confirmed that SLPW contributed to resistance to *S. aureus* and enhanced mouse survival. Unfortunately, the therapeutic effect of SLPW in uninfected control mice was temporary (**Figure 10**) and phage therapy before infection with *S. aureus* showed no effect on survival (data not shown).

The strength and efficiency of the host immune response depends on the level of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, which are indicators of the severity of infection (Wang et al., 2014; Lee et al., 2015). The results showed that phage therapy of uninfected mice (medium-treated groups) did not alter the cytokines levels (**Figure 11**). Therefore, phage treatment was considered safe in mice and cytokine experiments showed no bias. The IL-1β, IL-6, and TNF-α mRNA levels were significantly lower in the phage-administered mice (treatment groups) than in untreated mice (infected groups) at every time point (**Figure 11**). The results suggested that phage treatment successfully attenuated inflammation caused by *S. aureus* in mice.

The adverse effects associated with bactericidal agents include the release of large amounts of pathogen-associated molecular patterns recognized by Toll-like receptors and induction of pro-inflammatory cytokines in mammals (Ginsburg, 2002; Horner and Raz, 2003). However, our studies showed that treatment of uninfected mice with phage SLPW showed no significant differences in the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) during 24 h (**Figure 11**). In addition, phage therapy of uninfected mice resulted in similar survival as mice injected with normal saline after 7 days (data not shown). Our findings suggest that therapy using phage SLPW was safe, although phage residues in tissues may influence normal microflora in the human body (Endersen et al., 2014). Therefore, active therapy using phage SLPW is expected to be effective in the treatment of severe systemic infection caused by *S. aureus*.

CONCLUSION

In conclusion, our study investigates a lytic phage SLPW, which exhibits a wide host range, strong lytic activity and relative stability under various conditions. Rodent studies demonstrate a protective role of the phage SLPW in mice against MRSA infection, suggesting a potential antimicrobial role. Controlled clinical studies are needed to investigate the findings in animal studies.

AUTHOR CONTRIBUTIONS

JS, YY, ZW, and PZ designed experiments; ZW and PZ carried out experiments; ZW, WJ, QF and HW analyzed experimental results; ZW and JS wrote the manuscript.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00934

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In vitro Effectiveness of Commercial Bacteriophage Cocktails on Diverse Extended-Spectrum Beta-Lactamase Producing *Escherichia coli* Strains

Aycan Gundogdu[1,2], Darajen Bolkvadze[3] and Huseyin Kilic[1]*

[1] Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Erciyes University, Kayseri, Turkey, [2] Genome and Stem Cell Center (GENKOK), Erciyes University, Kayseri, Turkey, [3] Laboratory of Molecular Biology, G. Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilisi, Georgia

The objective of this study is to determine the *in vitro* susceptibility of Georgian bacteriophage cocktails on multidrug resistant (MDR) extended-spectrum beta-lactamase producing *Escherichia coli* (ESBL-EC) isolated from patients' blood and urine cultures. A total of 615 *E. coli* isolates were included in this study. Phene Plate (PhP)-typing and phylogenetic grouping were used for the typing. Antimicrobial resistance profiles and ESBL production of all isolates were confirmed according to Clinical and Laboratory Standards Institute (CLSI) criteria. The activities of four bacteriophage cocktails (Enko-phage, SES-bacteriophage, Pyo-bacteriophage, and Intesti-bacteriophage) were determined against 142 ESBL-EC using *in vitro* spot tests. According to this, Enko-phage were active against 87.3% of the tested strains while that ratio was 81.7% for Intesti-bacteriophage, 81.7% for Pyo-bacteriophage, and 59.2% for SES-bacteriophage cocktails. Based on the contingency tests, the phage cocktails were observed to be statistically significantly ($p < 0.001$) more effective on ESBL-EC strains belonging to phylogenetic groups D and B2. The employed phage cocktails were found to be affective against all tested resistant types. These results are promising especially for the infections that are caused by MDR pathogens that are difficult to treat. As this is a preliminary step to the potential clinical trials to be designed for the country, *in vitro* confirmation of their success on a MDR ESBL-EC collection should be accepted as an initial action, which is encouraging to consider clinical trials of phage therapy especially in countries which are not introduce phage therapy.

Keywords: ESBL-*E. coli*, bacteriophage, antibiotic resistance, phage therapy, phylogenetic grouping

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****Correspondence:***
Aycan Gundogdu
agundogdu@erciyes.edu.tr

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INTRODUCTION

Escherichia coli is the leading bacterial pathogens responsible for intestinal and extraintestinal infections, including urinary tract infections, bacteremia, and meningitis (Kaper et al., 2004). Certain strains of *E. coli* have been shown to carry certain genes coding for enzymes that hydrolyze a wide range of beta-lactams and are referred to as extended-spectrum beta-lactamase producing

E. coli (ESBL-EC). In recent years, the dissemination of ESBL-EC has become a serious health problem worldwide including Turkey (Pitout and Laupland, 2008; Rodriguez-Bano and Pascual, 2008; Tasbakan-Isikgoz et al., 2011; Hawser et al., 2012). ESBL producing pathogens are known to be resistant not only first, second, and third generation cephalosporins and monobactams, but also to several other classes of antibiotics. As a result, infections that are difficult to treat occur, and occasionally the use of "antibiotics of the last resort" such as carbapenems, colistin is inevitable (Cantón et al., 2012). However, unfortunately, emergence of resistance against these drugs of last resort is also observed around the world (Schwaber and Carmeli, 2008; Hasman et al., 2015). The upcoming threat of increase in the prevalence of untreatable infections has motivated the quest for alternatives of antibiotic therapy.

Bacteriophages (phages) are natural parasites of bacteria and they have been considered as effective agents for the treatment of bacterial infections since 1920s (Sulakvelidze et al., 2001; D'Herelle, 2007). The reason phages have been used as antimicrobial agents in that they can recognize, bind onto, and reproduce within a bacterial host, resulting in rapid cell lysis. However, poor understanding of phage biology and inconsistent outcomes experienced in early trials resulted in the phage therapy never being adopted universally (Sulakvelidze et al., 2001; Kutter et al., 2010). The advancement and consequently widespread use of antibiotics left phage therapy remaining as a common practice confined in the former Iron Curtain Countries where it has been successfully applied for around 90 years. Currently, although antibiotic therapy is successful in the treatment of majority of infections, frontline therapies are not effective in noticeable amount exceptions (Gould, 2009; Gootz, 2010; Keske et al., 2014) which is the underlying reason for the revitalization of phage therapy in bacterial infections around the world (Parracho et al., 2012). Thus, adopting this renewed approach we designed this preliminary study on ESBL-EC since it is a significant infection control and treatment challenge in the world.

This study aimed to examine the *in vitro* activity of Georgian bacteriophage cocktails which are used as part of standard clinical practice in the Republic of Georgia on a well-characterized ESBL-EC isolated from Turkish patients' blood and urine. As this is a first step to the potential clinical trials to be designed for the countries that have reached a very high prevalence country wide, the study is of capital importance.

MATERIALS AND METHODS

Sample Collection

Clinical samples belonging to five experimental groups were included in this study. Experimental groups are (i) the blood of hospitalized patients, (ii) the urine of hospitalized adult patients, (iii) the urine of hospitalized pediatric patients, (iv) the urine of adult outpatients, and (v) the urine of pediatric outpatients. All samples are collected from the patients attending a 1300-bed tertiary hospital in Kayseri, Turkey, between 2014 and 2016. The samples did not include descriptive information from any patients. Identification of *E. coli* strains was performed using conventional microbiological methods and VITEK-2 (bioMérieux) automated system. Extraction of chromosomal DNA was done by growing a single colony of the isolates in Luria–Bertani (LB) broth overnight, collecting pellet in 100 ml sterile Milli-Q water, and boiling at 95°C for 15 min. *E. coli* strains were subjected to confirmatory tests using PCR amplification of the universal stress protein (*uspA*) gene as previously described by Chen and Griffiths (1998).

Typing of Isolates

Phene Plate (PhP) Fingerprinting

All strains were typed using a high-resolution biochemical fingerprinting method specifically developed for *E. coli* strains (PhPlate AB, Stockholm, Sweden; Landgren et al., 2005). Inoculation of the plates was done according to the manual instruction and the plates were incubated at 37°C. The rate of each reaction was evaluated by measuring the absorbance value in each well after 7, 24, and 48 h of incubation using a digital scanner. After the final scan, the PhPlate software was used to create absorbance (A620) data from the scanned plate images and the mean absorbance in each well over the three readings was calculated, yielding the biochemical fingerprint for each isolate. The biochemical fingerprints of the isolates were compared pairwise and the similarities among the isolates were calculated by the correlation (similarity) coefficient and clustered according to the unweighted pair group method with arithmetic averages (Sneath and Sokal, 1973; Saeedi et al., 2005). Isolate pairs having a similarity coefficient above the default identity level (0.975) of software was regarded as identical and assigned to the same biochemical phenotype. All data handling, including calculations of correlations and coefficients as well as clustering were performed using the PhPlate software version 4002 (PhPlate AB, Stockholm, Sweden).

Phylogenetic Grouping

Phylogenetic grouping (A, B1, B2, D) was done for ESBL-EC strains using multiplex PCR with primers coding for *chuA* and *yjaA* genes and the DNA fragment TSPE4.C2 according to Clermont et al. (2000).

Strains belonging to the same biochemical phenotype and phylogenetic groups were considered as members of the same clone and were regarded as common types, and those differing by one method were regarded as single types.

Phenotypic ESBL Detection and Antimicrobial Resistance Test

ESBL production of the isolates was screened by the double-disk synergy on Mueller-Hinton agar using cefotaxime, ceftazidime, cefpodoxime, and ceftriaxone with and without clavulanic acid (10 mg), according to Clinical and Laboratory Standards Institute (CLSI), recommendations (Clinical and Laboratory Standards Institute [CLSI], 2015). After phenotypic confirmation, the susceptibility of ESBL-EC producing strains was tested against nine antimicrobial agents using Kirby–Bauer disk diffusion methods according to CLSI guidelines. Antimicrobial

agents included were ampicillin/sulbactam, ciprofloxacin, gentamicin, sulfamethoxazole, amoxicillin/clavulanate, piperacillin/tazobactam, amikacin, cefepime, imipenem, and ertapenem. *E. coli* ATCC 25922 and ESBL-positive $bla_{ctx-m-15}$ ESBL-producing strains were used as quality control strains.

In vitro Susceptibilities of Bacteriophage Cocktails

Among all ESBL-EC, one representative from each PhP-common type and all single types are included in phage assay. Spot tests were performed to determine the activities of four commercially available bacteriophage cocktails on each non-replicating ESBL-EC isolate. The bacteriophage cocktails produced in the George Eliava Institute and they were commercially available in sterile media containing $1 \times 10^{5-6}$ phage particles in 1 mL for Pyo-bacteriophage and Intesti-bacteriophage in Georgia. For the remaining two cocktails, the plaque forming unit/mL concentration is not available.

For spot test, all ESBL-EC isolates were initially tested for their purity on MacConkey agar no. 3. One single colony were transferred onto LB agar and incubated at 37° for 18 h. These steps were repeated twice to yield desired colonies (i.e., round shaped) with similar morphology in the agar plates. Single colonies were picked and incubated in LB broth for about 3 h. Then, 100 μL of the bacterial culture (A600 ca. 0.5) mixed with 5 ml of the molten semi-solid LB agar (0.6%) and over laid on the surface of the LB agar. After 10 min, 10 μL of phage suspension was applied on the bacterial layer and incubated at 37°C overnight. Bacterial sensitivity to phage preparations was established by lysis at the spot where the phage was deposited (Gabrilovich, 1973). Observing confluent, semi-confluent, opaque lysis or individual plaques determined the isolate susceptibility. When the lysis was not possible, the corresponding isolates were determined to be resistant. Each test was repeated thrice.

The following four commercial phage cocktails were included in this study.

Enko-phage active against *Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella heidelberg*, *Salmonella newport*, *Salmonella cholerae*, *Salmonella oranienburg*, *Salmonella dublin*, and *Salmonella anatum*; Shigella flexneri (serovars 1, 2, 3, 4) and Shigella sonnei (six different serovars), enteropathogenic E. coli, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Staphylococcus saprophyticus*.

SES-bacteriophage active against Staphylococci (*S. aureus*, *S. epidermidis*, and *S. saprophyticus*); Streptococci (*Streptococcus pyogenes*, *Streptococcus sanguis*, *Streptococcus salivarius*, and *Streptococcus agalactiae*) and different serotypes of entero-pathogenic *E. coli* serovars.

Pyo-bacteriophage active against *Staphylococcus* spp., *Streptococcus* spp, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris*, and *E. coli*.

Intesti-bacteriophage active against *Shigella* spp., *Salmonella* spp., *E. coli*, *Staphylococcus* spp., *Enterococcus* spp., *Proteus* spp., *P. aeruginosa*.

RESULTS

A total of 615 *E. coli* were isolated from urine ($n = 433$) and blood ($n = 182$) cultures of patients (one isolate per patient) included in each experimental group (**Table 1**). One hundred and seventy-five out of 615 *E. coli* strains were confirmed to be genuine ESBL producers. All 175 ESBL-EC isolates were grouped into 20 common types (i.e., types with more than two identical isolates) and 122 single types based on typing methods. The lowest number of identical isolates in a common type was 2, and the highest number of identical isolates belonging to a common type was 5. With the consideration of clonality the number of representative isolates was determined to be 142 for ESBL-EC. Phylogenetic distribution of these 142 strains revealed that, 40.8% of the isolates belonged to group D, 38.1% belonged to B2, 14.2% belonged to group A, and 7% belonged to B1.

The rates of ESBL-EC within each experimental group were found to be ranging between 14.7% (for urine culture of children outpatients) and 40.4% (for blood culture of hospitalized patients) (**Table 2**). In addition to ESBL production all isolates were found to be multidrug resistant (MDR, resistant to >3 classes; Magiorakos et al., 2012; **Table 3**).The rate of Carbapenem non-susceptible (non-S) strains was found to be ranging between 2.2 and 5.1% among different experimental groups of ESBL-EC.

All 142 non-replicating ESBL-EC were included in the bacteriophage assay. According to phage susceptibility assay 131 (92.3%) of the ESBL-EC strains were determined to be susceptible to at least one phage cocktail. Among those, Enko-phage, Intesti-bacteriophage, and Pyo-bacteriophage preparations were active against greater number of isolates than SES-bacteriophage (87.3, 81.7, 81.7, and 59.2%, respectively). Resistance and sensitivity profiles of these four phage preparations are given in **Table 4**.

Considering the phage susceptibility distributions of the tested strains with respect to different phylogenetic groups, it was observed that each phylogenetic group shows different phage susceptibility characteristics. While Enko, Pyo, and Intesti phages are dominantly effective on B2 and D phylogenetic groups (7.4% resistance rate for all phage cocktails in B2, 3.4, 5.2, and 8.6% resistance rates for Enko, Pyo, and Intesti, respectively in D), a more even susceptibility response can be observed for A and B1

TABLE 1 | Numbers of confirmed *E. coli* strains included in this study within each experimental group.

Experimental groups	Number of *E. coli* strains		
	ESBL −ve	ESBL +ve	Total
Urine			
Hospitalized adults	74	43	117
Hospitalized children	26	19	45
Adult outpatients	92	26	118
Children outpatients	131	22	153
Blood (adults and children)	117	65	182
Total	440	175	615

ESBL −ve, strains not producing extended-spectrum beta-lactamases; ESBL +ve, strains producing extended-spectrum beta-lactamases.

TABLE 2 | Distribution of 615 *E. coli* strains within each experimental group after PhP-typing.

Experimental groups	Number of representative strains				PhP-types		
	ESBL −ve	ESBL +ve	Total	%ESBL +ve	Common types	Single types	DI
Urine							
Hospitalized adults	65	33	98	33.7	12 (31)	86	0.996
Hospitalized children	23	14	37	37.9	5 (13)	32	0.991
Adult outpatients	70	21	91	23.01	18 (45)	73	0.994
Children outpatients	87	15	102	14.07	25 (76)	77	0.990
Blood	87	59	146	40.4	23 (59)	123	0.996

ESBL −ve, strains not producing extended-spectrum beta-lactamases; ESBL +ve, strains producing extended-spectrum beta-lactamases; DI, diversity index. The numbers in the bracket indicated how many strains belong to common types.

TABLE 3 | Antimicrobial resistance percentages of 142 ESBL-EC strains.

Experimental groups	Antimicrobial agents tested									
	SAM	CIP	GEN	STX	AMC	TZP	AMK*	FEP	IMI	ERT
Urine (83)										
Hospitalized patients (47)	82	73.3	42.3	74.5	51.2	30	5	100	2.2	2.2
Outpatients ($n = 36$)	79.2	60.5	24.4	60.2	47.6	20	0	100	0	0
Blood ($n = 59$)	95	67.8	48	78	59.5	40.7	3.4	96.6	5.1	3.4

*SAM, ampicillin/sulbactam; CIP, ciprofloxacin; GEN, gentamicin; STX, sulfamethoxazole; AMC, amoxicillin-clavulanate; TZP, piperacillin/tazobactam; AMK, amikacin; FEP, cefepime; IMI, imipenem; ERT, ertapenem. *Reduce susceptibility.*

phylogenetic groups ($p < 0.00001$, chi-square test). This trend can also be observed for SES cocktail with statistical significance ($p = 0.000295$, chi-square test), yet effectiveness for B2 and D phylogenetic groups seems to be less than the other cocktails (27.7 and 34.4% resistance rates for B2 and D groups, respectively) (**Table 5**).

ESBL-EC strains isolated from hospitalized patients were more susceptible to Enko-phage than other phage preparations, whereas outpatients' isolates were more susceptible to Pyo-bacteriophage than the other cocktails. Eleven (7.7%) of the ESBL-EC isolates were found to be resistant to all commercially available bacteriophage cocktails. These 11 ESBL-EC were isolated from blood ($n = 5$), from the urine of hospitalized adults ($n = 2$), from the urine of pediatric outpatients ($n = 2$) and from the urine of adult outpatients ($n = 2$). The susceptibility/non-susceptibility patterns of *E. coli* showed no correlation with their antibiotic resistance patterns.

TABLE 4 | Sensitivity profiles of tested phage cocktails on 142 ESBL-EC included in this study.

	Number of ESBL-EC strains			
	ENKO	SES	PYO	INTESTI
Confluent	38	1	38	35
Semi-confluent	29	10	27	20
Opaque lysis	23	7	23	27
Individual plaques	34	66	28	34
Resistant	18	58	26	26

ENKO, Enko-phage; SES, SES-bacteriophage; PYO, Pyo-bacteriophage; INTESTI, Intesti-bacteriophage.

DISCUSSION

Over the past decade, the prevalence of ESBL-EC has attracted the attention of health authorities due to their increasing rates on a daily basis (Paterson and Bonomo, 2005). Recent studies have shown that the prevalence of ESBL-EC in different countries can be as high as 63%, with the highest rate belonging to *E. coli* isolated from ICU patients in Turkey (Hawser et al., 2009; Magiorakos et al., 2012; Nakai et al., 2016). Reports indicated that ESBL-EC rates are generally very high in Turkey; however, previous studies generally do not take the clonal relationships of the isolates into account (Kacmaz et al., 2005). Although molecular typing methods such as pulsed field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) have been used extensively for determining clonal relationships of the investigated strains, for practical purposes, we employed the PhP-typing method that specifically developed to discriminate *E. coli* strains (Kühn et al., 1995). This system has been used in several epidemiological studies (Ahmed et al., 2006; Cassanovas-Massana and Blanch, 2013) and was shown to have similar discriminatory power with the genotypic methods (Ansaruzzaman et al., 2000; Vollmerhausen et al., 2011). Thus, the ESBL-EC rates (between 14.7 and 40.4%) reported in this study might be indicating a more accurate estimate for Turkish outpatients and hospitalized patients. However, the ESBL-EC ratio especially for hospitalized patients, are still very high and the required precautions should be taken urgently. In addition to ESBL production, the high antibiotic resistance

TABLE 5 | Association between phylogenetic groups and phage resistance among 142 ESBL-EC strains.

	Number of strains	Number of strains in each phylogenetic groups				
		B2	D	A	B1	*p* value
ENKO						
Resistant	18	4	2	10	2	<0.00001
Susceptible	124	50	56	10	8	
SES						
Resistant	58	15	20	17	6	0.000295
Susceptible	84	39	38	3	4	
PYO						
Resistant	26	4	3	14	5	<0.00001
Susceptible	116	50	55	6	5	
INTESTI						
Resistant	26	4	5	13	5	<0.00001
Susceptible	116	50	53	7	5	

ENKO, Enko-phage; SES, SES-bacteriophage; PYO, Pyo-bacteriophage; INTESTI, Intesti-bacteriophage; p values were calculated using chi-square test.

rates found this study might be attributed to their frequent preference in empirical treatment and the overall antibiotics usage policies. A previous Turkish meta-study highlighted the increase of ciprofloxacin, cefepime, co-trimoxazole resistance and ESBL production during the study period of 1996–2012 (Aykan and Cifci, 2013). As co-resistance to aminoglycosides and quinolones is developed quickly during the ESBL-EC treatments, carbapenems have been the drugs of choice when an infection is caused by ESBL-EC. In this study carbapenem resistance ratio was found to be between 2.2 and 5.1%. Since the carbapenems are still active against the majority of ESBL-EC, clinicians increasingly prefer carbapenems for empiric or definitive therapy in community-onset and nosocomial infections caused by this pathogen (Rodríguez-Bano et al., 2012). However, this situation results in a frequent explosion of carbapenems to bacteria, which consequently makes the spreading of carbapenemase-producing bacteria scenario more probable and worrisome.

The treatment difficulties on infections caused by MDR pathogens continue to be a vexing problem. Thus, alternative approaches as the treatment options are in quest. Being one of the most popular approaches employing phages as anti-infective agents was adopted in this study. The results of the study indicate that, a great majority (131/142, 92.3%) of diverse ESBL-EC strains were susceptible to at least one phage cocktail. Furthermore, considering the high diversity among each experimental groups based on typing methods, it might be concluded that phages are effective on a broad range of ESBL-EC clones. This is quite promising especially from the point of infections difficult to treat that are caused by MDR pathogens. In an Irish study conducted by Fitzgerald-Hughes et al. (2014), 82% of ESBL-EC isolated from patients were found to be susceptible to the same bacteriophage cocktails used in current study. Higher percentage found in our study might be explained by the geographical contact between Georgia and Turkey. Because of the neighboring geographies, residents go back and forth between these two countries resulting in bacterial transfers. Thus, as the producer (The G. Eliava Institute of Bacteriophages, Microbiology and Virology) updates its commercial phages regularly, new phages against new strains disseminated from Turkey (e.g., via patients, food, etc.) might find a room in these bacteriophage cocktails. However, in another study conducted by Sybesma et al. (2016) in which they used 29 *E. coli* bacteriophages from the Eliava collection in addition to four commercial phage cocktails on *E. coli* set compromising ESBL negative and positive *E. coli*, the phage susceptibility rate was found to be 92.6%. In the current study, 7.7% ESBL-EC isolates were found to be resistant to all bacteriophage cocktails tested. Since the phage cocktails are originated from Georgian biogeography, the phages of these strains might not be contained in the cocktail preparations. On the other hand, isolation of bacteriophages, which are able to be effective on these resistant strains from those pathogens' own environment might be possible. With a specified preparation of phage cocktails and/or with the adaptation of bacteriophages (Sybesma et al., 2016), even more successful susceptibility results are potentially achievable. In our study, SES-bacteriophage was the least active (59.2%) cocktail whereas in Fitzgerald-Hughes et al.'s (2014) study, SES-bacteriophage was the most active phage preparation. This outcome might be the indication of different *E. coli* sub-groups being common in Ireland and in Turkey. According to our study, bacteriophage cocktails were found to be effective against all antimicrobial resistance types tested, including carbapenem resistance. This orthogonality between the phage activity and the antimicrobial resistance might be an encouraging factor for the adoption of phage therapy as an alternative or complementary option to difficult to treat infections. For instance, in this study the majority of ESBL-EC (58.4%) were isolated from the urine cultures of the patients. For these patients, carbapenems are generally considered as the drug of choice (Prakash et al., 2009) and in Turkey there are no oral options for carbapenem treatment. Therefore, treatment for the urinary tract infections caused by ESBL-EC generally requires hospitalization due to intravenous antibiotic therapy application. This is an unfavorable situation as it increases the odds of the patients' being exposed to nosocomial infections, as well as increasing the medical expenses. Considering these multiple drug resistant pathogens are frequently susceptible to the tested phages according to our *in vitro* experiments, employing phage therapy (oral) with *in vitro* tested phage preparations could be an alternative or complementary approach for the cases that narrow spectrum antibiotic therapy is not sufficient or hospitalization is required.

The association of the phylogenetic groups with the virulence characteristics of *E. coli* has been repeatedly reported in the literature, which allows a separation of commensal (A and B1) and pathogenic (B2 and D) strains (Bingen et al., 1998; Duriez et al., 2001). As B2 and D are mainly accepted to contain the pathogenic strains, while A and B1 phylogenetic groups are known to mostly include environmental/commensal strains, the altering characteristics might reveal a feature for the employed phage cocktails. According to our results, the cocktails have a particular effectiveness on the pathogenic strains. The

underlying reason for that phenomenon might be an artificial selection of "pathogen killing" bacteriophages during phage adaptation procedures since they are selected on clinical strains. Anyhow, this finding can lead us to draw a conclusion that the bacteriophage cocktails are highly specific to selected pathogens, which might turn out to be a preferable feature as they are less likely to harm beneficial components of human microbiome even when they are used orally.

Journals published since 1920s have indicated that the success rate of the *in vivo* phage therapy—orally, rectally, locally, intrapleural injections or intravenously, etc.—is nearly 95%, clinical improvements or full recovery is usually observed within at most 7 days with no reports of serious complications or side effects (allergy, kidney or liver failure, etc.) associated with their use (Chanishvili, 2012). Although clinical practice of phage therapy as a common medical practice has not been approved yet, except a few Eastern European countries, some studies conducted in EU have already reached the clinical trial phase (Merabishvili et al., 2009; Rhoads et al., 2009; Wright et al., 2009; Pelfrene et al., 2016). As an example, a study started in 2013 and conducted within European Union PF7 programme with the collaboration of researchers from Belgium, France, and Switzerland, achieved their first phage therapy application of burn wound infections on human patients in July 2015[1].

CONCLUSION

This study demonstrated the *in vitro* activity of bacteriophage preparations that are used as a part of standard clinical practice in the Republic of Georgia, against MDR ESBL-EC isolated from Turkish patients. Although bacteriophage therapy has been a successful part of standard healthcare practice for decades, the non-practicing camp has been skeptical to accept bacteriophages as alternative anti-infectives. However, the need for novel antimicrobial therapies have always been of great importance, yet with the emergence of pan-resistant Gram-negative pathogens recently (Arias and Murray, 2009; Glupezynski et al., 2010) it could be argued that the quest has never been that crucial since the beginning of the antibiotic era. Thus, *in vitro* confirmation of their success on a well-characterized isolate collection is reported as an initial action, which is encouraging for their clinical use especially for infections with treatment difficulties. Moreover, the effectiveness of generic phage cocktails which are not specifically produced for strains of concern is an implication that the spectrum of phages is large enough to allow their general use.

[1] http://www.phagoburn.eu

AVAILABILITY OF DATA AND MATERIALS

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

AG conceived and designed the study, performed the tests, analyzed and interpreted the data, and wrote the manuscript. HK collected the strains and performed the automated identification and antimicrobial susceptibility tests. DB participated in the phage study and writing and revision of the manuscript. All authors read and approved the final manuscript.

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Small Colony Variants and Single Nucleotide Variations in Pf1 Region of PB1 Phage-Resistant *Pseudomonas aeruginosa*

Wee S. Lim[1,2,3], Kevin K. S. Phang[1], Andy H.-M. Tan[1,4], Sam F.-Y. Li[2,3,5] and Dave S.-W. Ow[1]**

[1] Agency for Science, Technology and Research, Bioprocessing Technology Institute, Singapore, Singapore, [2] NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore, Singapore, [3] NUS Environmental Research Institute, National University of Singapore, Singapore, Singapore, [4] Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore, [5] Department of Chemistry, Faculty of Science, National University of Singapore, Singapore, Singapore

Phage therapy involves the application of lytic bacteriophages for treatment of clinical infections but bacterial resistance may develop over time. Isolated from nosocomial infections, small colony variants (SCVs) are morphologically distinct, highly virulent bacterial strains that are resistant to conventional antibiotics. In this study, SCVs was derived from *Pseudomonas aeruginosa* exposed to the lytic bacteriophage PB1 and these cells were resistant to subsequent phage infection by PB1. To elucidate the mechanism of the SCV phage resistance, we performed phenotypic assays, DNA microarrays and whole-genome sequencing. Compared with wild-type *P. aeruginosa*, the SCV isolate showed impaired biofilm formation, decreased twitching motility, reduced elastase and pyocyanin production. The SCV is also more susceptible to the antibiotic ciprofloxacin and exhibited higher syrface hydrophobicity than the wild-type, indicative of changes to cell surface lipopolysaccharide (LPS) composition. Consistent with these results, transcriptomic studies of SCV revealed up-regulation of genes involved in O-specific antigen (OSA) biosynthesis, suggesting the regulation of surface moieties may account for phage resistance. Western blot analysis showed a difference in OSA distribution between the two strains. Simultaneously, genes involved in aromatic and branched chain amino acid catabolism were down-regulated. Whole genome sequencing of the SCV revealed multiple single nucleotide variations within the Pf1 prophage region, a genetic locus known to play a crucial role in biofilm formation and to provide survival advantage via gene transfer to a subpopulation of cells. Insights into phenotypic and genetic changes in SCV gained here should help direct future studies to elucidate mechanisms underpinning phage resistance, leading to novel counter resistance measures.

Keywords: ***Pseudomonas aeruginosa*, phage therapy, phage resistance, small colony variants, Pf1 region**

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****Correspondence:***
Sam F.-Y. Li
chmlifys@nus.edu.sg;
Dave S.-W. Ow
dave_ow@bti.a-star.edu.sg

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INTRODUCTION

The emergence of multidrug-resistant pathogens and the difficulties in developing new antibiotics have spurred the resurgence of interest in phage therapy, involving the use of lytic bacteriophages against specific pathogens as a form of treatment (DiMasi et al., 2003; Gilbert et al., 2003; Spellberg et al., 2004; Talbot et al., 2006; Keen, 2012; Ly-Chatain, 2014). *Pseudomonas aeruginosa* is an opportunistic pathogen found ubiquitously in urban and natural environments, e.g., soil, rivers and sewage. In hospital settings, bacteria colonizes the surfaces of medical equipment such as catheters, inhalers, nebulizers and tubings (Stamm, 1978; Kirschke et al., 2003), where they account for approximately 10% of nosocomial infections (System, 2004). *P. aeruginosa* have been isolated from urinary tract and inner ear infections, burn wounds and from the surface of human epithelia in cystic fibrosis patients (Govan and Deretic, 1996; Lyczak et al., 2000; Lang et al., 2004; Taneja et al., 2004). Eradicating *P. aeruginosa* is not trivial as it has evolved various resistance mechanisms against conventional antibiotic therapies (Yoshimura and Nikaido, 1982; Nickel et al., 1985; Poole, 2004). Phage therapy has thus gained increasing consideration as an alternative treatment for antibiotic-resistant bacteria. Currently, phage therapies against methicillin-resistant *Staphylococcus aureus* and pathogenic *Escherichia coli* are in clinical trials (Harper and Enright, 2011). Studies have been carried out to elucidate how *P. aeruginosa* affects animal models of gut sepsis (Watanabe et al., 2007), burn wound (McVay et al., 2007) and lung infection (Morello et al., 2011). In one human clinical trial, Wright et al. (2009) administered a bacteriophage cocktail to treat chronic otitis. In another, Khawaldeh et al. (2011) reported the use of a lytic bacteriophage cocktail to treat a human patient suffering from *P. aeruginosa* urinary tract infection. Although these reports indicate that while phage therapy can be initially effective against *P. aeruginosa*, spontaneous phage resistance often occurs afterward, rendering the phage therapy ineffective.

Small colony variants (SCVs) of infectious strains of bacteria were first identified in 1910 (Proctor et al., 2006), so named because the colonies formed by SCVs are one 10th the colony size of their wild-type counterparts. SCVs exhibit higher antibiotic resistance than wild-type bacteria and they often form after exposure to high concentrations of antimicrobial agents such as gentamicin (Wei et al., 2011). Studies have also showed that SCVs could be induced in planktonic cultures of *P. aeruginosa* in response to infection by the lysogenic filamentous phage Pf4 (Webb et al., 2004; Hui et al., 2014). In the current study, a phage resistant SCV (F1 strain) of *P. aeruginosa* PAO1 strain (F0 strain) was successfully isolated using the lytic phage PB1. The first PB1 phage was first described in Holloway et al. (1960). Subsequently, a family of at least 42 other PB1-like bacteriophages against *P. aeruginosa* was discovered (Krylov et al., 1993; Pleteneva et al., 2008; Ceyssens et al., 2009). PB1 and PB1-like bacteriophages belong to the *Myoviridae* phage family and use bacterial lipopolysaccharide (LPS) as their receptor (Kropinski et al., 1977), and these lytic bacteriophages are a family of promising agents for phage therapy(Garbe et al., 2010; Krylov et al., 2013). Phage cocktail containing PB1-like *myoviridae* phages are currently use in clinical trials (Kwan et al., 2006; Merabishvili et al., 2009). The selection pressure imposed by PB1 phage allowed the isolation of SCVs which produce smaller colonies than their wild-type counterparts on agar plates. Besides determining the SCVs' resistance to subsequent PB1 infections other characteristics such as their surface hydrophobicity, pyocyanin production, biofilm formation and cell lengths using microscopy were determined as well. The gene expression profiles of both wild-type and SCV *P. aeruginosa* were studied using DNA microarrays, and several pathways that could potentially confer phage resistance in SCV were identified. Whole genome sequencing enabled identification of point mutations and single nucleotide polymorphisms in the genome of SCVs that could have conferred a survival advantage and resulted in other phenotype changes in the SCVs.

MATERIALS AND METHODS

Bacterial Strains and F1 Strain Isolation

Pseudomonas aeruginosa strain PAO1 (ATCC 47085) was designated as the wild-type F0 strain in this work. Glycerol stock of F0 was streaked on LB agar plates supplemented with 10 μg/mL tetracycline and incubated overnight at 37°C. For sub-culturing, 1 mL of overnight culture was added to 25 mL of LB broth diluted with 25 mL of reduced strength LB (20%) broth and incubated at 37°C, 225 rpm for all experiments unless otherwise stated. For phage infection, 500 μL of PB1 phage stock (1×10^{10} PFU/mL) was added to the subculture after allowing the subculture to recover at 37°C, 225 rpm for 1 h. Infected cultures were cultured for 24 h at 37°C, 225 rpm. The culture was streaked on fresh LB plates with 10 μg/mL tetracycline and incubated overnight at 37°C. The SCV was isolated (F1) for subsequent experiments.

Determination of the Stability of SCV Phenotype

Single colonies of F0 and F1 were inoculated in 5 mL LB media and incubated at 37°C, 225 rpm for 6 h. The cultures were streaked onto agar plates and incubated at 37°C overnight. The colony size of both F0 and F1 were compared the following day. The SCV phenotype was determined to be stable as long as the colony size of F1 remained smaller than that of F0. The process was repeated for seven passages.

OD600 Measurements, Cell Viability Assays, Generation Time Determination and Gram Staining and Microscopy

OD reading was measured at 600 nm using a UV-vis spectrophotometer in a 1 cm cuvette. Serial dilutions (10^{-1} to 10^{-7}) of cultures were performed in 0.01% peptone. 100 μL of 10^{-3} to 10^{-7} dilutions with duplicates were spread on LB plates with 10 μg/mL tetracycline. Plates were incubated overnight at 37°C. Plates with colonies ranging from 25 to 300 were counted and the numbers obtained averaged. Morphologies on agar plates

were observed. For the determination of generation time, hourly OD600 measurements were taken for 7 h and a growth curve was plotted (not shown). By measuring the gradient for the linear portion of the plot, the generation time could be determined. For microscopy studies, F0 and F1 cultures at mid-log were heat fixed and Gram-stained (Sigma) according to manufacturer's protocol. The slides were viewed under a 100X objective with an Eclipse Ni-U microscope (Nikon) under oil immersion.

Antibiotic Susceptibility Measurement with Etest

One mililiter of an overnight culture was diluted in 50 mL of LB media and incubated at 37°C, 225 rpm for 4 h. The turbidity of the culture was adjusted with 1X PBS to match that of MacFarland standard 0.5. Bacterial lawn of F0 or F1 was streaked onto Mueller-Hinton II agar plates with a cotton swab and plates allowed to dry for 5 min. An Etest strip (bioMérieux) containing gentamicin or ciprofloxacin was then placed onto the agar plate with sterile tweezers and the plates were incubated at 37°C overnight. The minimum inhibitory concentration (MIC) readings were read off at the point where the inhibition ellipse intersects the scale on the strip.

Biofilm Formation Assay

The assay was adapted from the original protocol as described by Stepanović et al. (2000). A single colony of bacteria was inoculated in 5 mL Vogel-Bonner medium supplemented with 2% (w/v) glucose and incubated overnight at 37°C. Two hundred microliter of the overnight culture was seeded per well into a 96-well plate and the plate was further incubated for 24 h at 37°C. The culture was carefully aspirated with a pipette and each well was washed once with 200 μL of 1X sterile phosphate buffer saline (PBS), pH 7.5. The plate was inverted and dabbed dry onto paper towels after washing. One hundred and fifty microliter of methanol was added per well to fix the cells for 7 min and the methanol was then removed by removed by inverting the plate and gently flicking off any residual liquid. The plate was then dabbed dry onto paper towels. One hundred and fifty microliter of crystal violet was added per well to stain the attached cells for 30 min and the crystal violet was then removed by inverting and gently flicking the plate. The wells were then rinsed gently with tap water. The plate was inverted and finally dabbed dry onto paper towels. The amount of attached cells was then quantified via the solubilization of the bound crystal violet through the addition of 150 μL of 33% acetic acid into each well. The absorbance at 620 nm (A_{620}) was measured using a plate reader.

Elastase and Pyocyanin Assays

The assay was modified from the procedure given by Kamath et al. (1998) and Carlsson et al. (2011). Bacteria were grown for 60 h in King's Medium A (King et al., 1954). Five mililiter of the overnight culture was centrifuged at 4600 rpm for 5 min at room temperature and the supernatant was collected and filtered through a 0.2 μm filter.

To measure the specific activity of elastase, 20 mg of Elastin-Congo Red (Sigma) substrate was added to 2 mL of reaction buffer (30 mM Tris-HCl; pH 7.5) in a 15 mL falcon tube. After warming the mixture to 37°C, 800 μL of filtered supernatant was added. The entire mixture was further incubated at 37°C, 225 rpm for 2 h. Elastin-Congo Red was pelleted by centrifugation at 4600 rpm for 10 min and the OD495 of the supernatant was measured with reaction buffer as the blank. The background absorbance was determined by measuring the absorbance of the reaction buffer and filtered supernatant without Elastin-Congo Red. Measurements were carried out in triplicate by a Infinite 200 PRO plate reader (Tecan) and the values were averaged and corrected for background absorbance. The elastase activity (OD495 hr^{-1} mL^{-1}) of the filtered supernatant was calculated by adjusting the corrected OD495 values for incubation time, reaction volume and dilution factors. The amount of total protein in the filtered supernatant was determined with a Bradford assay, using the Coomassie Plus Assay Kit (Pierce) according to manufacturer's instructions. Finally, the specific activity of elastase (OD495 mg $protein^{-1}$ h^{-1}) in the supernatant was determined by dividing the elastase activity with the total protein concentration.

For the measurement of pyocyanin concentration, 2 ml of chloroform was added to the filtered supernatant and vortexed for 30 s. The mixture was then centrifuged at 4600 rpm for 10 min to allow complete separation of the aqueous and organic phases. Eight hundred microliter of the blue chloroform phase was collected and mixed with 200 μL of 0.2 M HCl before vortexing for 30 s. One hundred fifty microliter of the pink layer was added to a 96 well plate and OD520 was measured with Infinite 200 PRO plate reader (Tecan). OD520 was multiplied by a factor of 17.072 to obtain the concentration of pyocyanin and the concentration of pyocyanin in the supernatant was determined by taking into account of dilution factors.

Twitching Motility and Microbial Adhesion to Hydrocarbon (MATH) Assays

The twitching assay was carried out as described by Rashid and Kornberg (2000). Bacteria were stabbed onto twitch agar plates (1% agar LB plates). Twitch plates were incubated at 37°C for 48 h. The diameter of the zone of twitching was measured to estimation bacterial motility. To determine surface hydrophobicity, the MATH assay was adapted from the work of Pérez et al. (1998). Bacteria were first grown to mid-log in LB media. A 4 mL aliquot of the culture was centrifuged at 4600 rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in 4 ml of PBS. This aliquot was then sub-divided into 2 aliquots of 2 ml each. Four hundred microliter of xylene was added to one of the tubes. Both tubes were vortexed for 2 min before allowing the tubes to stand for 30 min. Subsequently, 1 mL of aqueous phase from each aliquot was added to a cuvette and the OD OD600 measured (OD_0 and OD_{xylene} representing the OD of the aliquot without and with xylene respectively). Surface hydrophobicity (H%) was calculated by the percentage change in turbidity before and after addition of xylene using the formula: $\frac{OD_{xylene} - OD_o}{OD_o} \times 100$

Statistical Analysis for Phenotypic Assays

The differences between F0 and F1 were assessed with the Mann–Whitney test (for cell length measurement as the distribution of cell length are not normally distributed) and the unpaired Student's *t*-test (for all other assays) using GraphPad Prism. Values are reported as mean ± standard deviation (SD). *P*-values < 0.05 were considered statistically significant.

LPS Extraction and Western Analysis

The LPS extraction procedure was modified from that of Davis and Goldberg (2012). A 5 mL suspension of mid-log culture (OD 0.5) was centrifuged at 4600 rpm for 10 min and the supernatant was discarded. The bacterial pellet was resuspended in 200 μL 1X SDS buffer (2% β-mercaptoethanol, 2% SDS, 10% glycerol in 0.1M Tris-HCl pH 6.8, pinch of bromophenol blue) and boiled for 15 min. The solution was allowed to cool to room temperature for 15 min and 10 μL of 10 mg/mL Proteinase K was added. Samples were then incubated at 59oC overnight. Two hundred microliter of ice cold Tris-saturated phenol was added and samples were vortexed for 5 s. The mixture was incubated at 65oC for 15 min with occasional vortexing and then allowed to cool to room temperature. One mililiter of diethyl ether was added and vortexed for 5 s centrifuging at 20,600 *g* for 10 min. The bottom layer containing the LPS was carefully removed by a pipette. The remaining solution was re-extracted a second time by adding 200 μL of ice cold Tris-saturated phenol and following the steps above.

For LPS Western blot analysis, 15 μL of each sample was run on a denaturing 4–20% Tris-Glycine gel (Novex) according to manufacturer's instructions. Monoclonal antibodies 5C7-4, 5c-101 and MF15-4 (MyBiosource) recognizing the inner, outer core and O-specific antigen (OSA) for O5 serotype were used as the primary antibody, and goat anti-mouse IgG-HRP (SantaCruz Biotechnology) was used as secondary antibody. Chemiluminescence detection was carried out using Clarity™ Western ECL blotting substrate (Biorad), and the blot was visualized on ImageQuant LAS 500 (GE Healthcare Life Sciences).

DNA Microarray

A total of four biological replicates of F0 and F1 each were used. Cells were grown to OD600 = 0.5 (mid-log) and 10 mL aliquots of culture were treated with 20 mL RNAProtect bacteria reagent (Qiagen) according to manufacturer's protocol. RNA was isolated from these cells using RNAeasy MIDI kit (Qiagen). RNA was then converted to cDNA using Superscript II (Invitrogen). The cDNA was fragmented using DNAseI (New England Biolabs) and then labelled with Genechip DNA labeling reagent (Affymetrix) and terminal deoxynucleotidyl transferase (Promega). The hybridization cocktail was prepared using the GeneChip Hybridization, Wash and Stain Kit (Affymetrix) and hybridized to a *P. aeruginosa* genome array (Affymetrix). All steps performed were carried out according to the respective manufacturers' protocol. Arrays were hybridized for 16 h at 50°C and then washed and stained with the GeneChip Hybridization, Wash and Stain Kit (Affymetrix). Data from the arrays were analyzed using Partek Genomics Suite. A list of differentially expressed genes (DEGs) was constructed using the criteria of $p < 0.05$ (one-way ANOVA) and a fold change (FC) of 1.5. Data are deposited in the Gene Expression Omnibus (GEO) database under accession number GSE75654.

Next Generation Sequencing (NGS)

The Puregene kit (Qiagen) was used to isolate genomic DNA from overnight bacterial cultures, according to manufacturer's instructions. The quality of the isolated gDNA was assessed through Nanodrop (Thermo Scientific). A total of five biological replicates for F1, four biological replicates for F0 were used. Genomic library preparation was carried out using Nextera XT kit (Illumina) and according to manufacturer's instructions. All samples were pooled and ran on Mi-Seq (Illumina) in a 2 × 300 run. Raw reads were trimmed and aligned to *P. aeruginosa* PAO1 reference genome (Stover et al., 2000) using online tools[1]. The sequences were also compared with the annotated genome of *P. aeruginosa* strain PAO1[2]. Aligned reads were analyzed using Partek Genomic Suite to identify single nucleotide variations (SNVs). Only SNVs with a coverage >50, non-reference average base quality >20, and non-reference average mapping quality >20 were retained to generate an SNV list.

RESULTS

SCV Phenotype of Derived Strain of F1

Wild-type *P. aeruginosa* PAO1 (F0) was cultured and infected with PB1 phage. After overnight incubation at 37°C and centrifugation at 225 rpm, the culture was streaked on the agar plate and further incubated. A number of morphologically distinctive small colonies was observed (**Figure 1A**) and a SCV was isolated (F1) from the plate. To check if SCV phenotype of F1 was stable, the cells were sub-cultured and plated on agar plates. **Figure 1A** shows the colony morphology of F0 and F1 strains after 24 h incubation at 37°C. F1 colonies consistently showed the typical small and transparent colonies of an SCV after subculturing and this phenotype persists after seven passages. It was hypothesized that there may be variations within the genome of F1 forming a genomic imprint that allows the SCV phenotype to be maintained. Since F1 was isolated under the selection pressure imposed by PB1 phage, it is possible that F1 is resistant to subsequent bacteriophage treatment. Both F0 and F1 were re-infected with PB1 and their cell viabilities at 0, 3, 6, and 24 h post infection were measured, using their uninfected counterparts as controls (**Figure 1B**). When F0 is infected with phage, its viability decreased after 3 h, then increased at 6 h and eventually attained 10^9 CFU/ mL after 24 h. In contrast, the viability of F1 increased steadily, in a manner similar to uninfected F0, to 10^9 CFU/mL after 24 h, regardless of whether phage was added. The generation time for F1 strain as determined by OD600 measurement is lower than F0 (**Figure 1C**), which may suggest that F1 has a faster growth rate, contradicting previous reports on SCVs being slow-growing (Proctor et al., 2006). However, cell

[1] usegalaxy.org

[2] www.pseudomonas.com

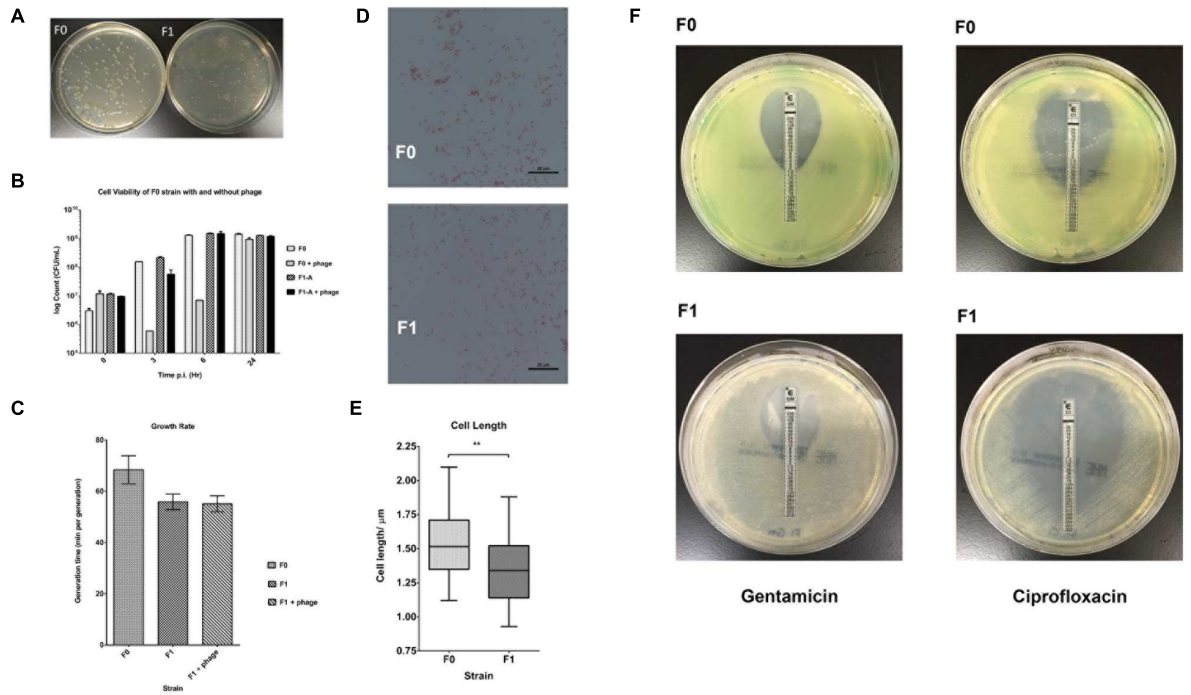

FIGURE 1 | Morphology and Cell Viability. (A) Bacteria was plated on agar plates and incubated for 24 h at 37°C; *left- F0 (wt); right- F1 (SCV)*. **(B)** Bacteria count at 0, 3, 6, 24 h post infection (p.i.) for F0 and F1 strains. **(C)** Generation times (time taken for the doubling of population) for F0 and F1 strains, a total of three biological replicates were carried out. **(D)** Morphologies of gram-stained F0 and F1 strains, viewed under a 100x objective. **(E)** Distribution of cell length of 40 replicates each for F0 and F1 strains; Mann–Whitney test. F1 cells are significantly smaller than F0 cells. **(F)** Antibiotic susceptibility of F0 and F1 strains to gentamicin (left panels) and ciprofloxacin (right panels). $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

viability measurements showed there is no difference in growth (**Figure 1B**). This is because F0 and F1 cells have different sizes, thus producing the dichotomy between optical density and cell viability (Sutton, 2011). A representative set of 40 cells from each of F0 and F1 strains were picked for cell length measurement. As shown in **Figures 1D,E**, F1 has a distribution of cell lengths which is noticeably shorter than that of F0. From the Etest results, there appears to be no difference in gentamicin susceptibility but an increase in ciprofloxacin susceptibility in F1 (**Table 1**; **Figure 1F**).

Several phenotypic assays (biofilm formation, elastase/pyocyanin assays, twitching motility, microbial adhesion to hydrocarbon or MATH assay,) were also carried out to characterize the other physiological differences between F0 wild-type and F1 SCV strains. Using crystal violet to quantify biofilm on 96-wells (**Figure 2A**), it was found that biofilm formed by F1 cells on the surface of the wells have a lower absorbance at 620 nm than F0; indicating a lower amount of biofilm formation by the F1 compared with the F0 cells. The elastase and pyocyanin assays (**Figures 2B,C**) detected lower amounts of elastase and pyocyanin in the supernatant of F1 cultures compared to F0. Since both elastase and pyocyanin are virulence factors whose functions include inhibiting the growth of competing microflora, damaging pulmonary tissues and contributing to *P. aeruginosa* persistence in cystic fibrosis patients (Lau et al., 2004), decreased production of these factors by the phage-resistant F1 could result in lesser adverse effects if phages were used to treat patients. The twitching assay (**Figure 2D**) showed that the twitching zone of F1 is smaller than that of F1, indicating that F1 cells have lower surface-associated motility than F0 in 1% agar. The MATH assay is a measurement of surface hydrophobicity, based on the partitioning of cells between organic and aqueous phases. H% of F1 is higher than that of F0 (**Figure 2E**), suggesting that F1 may have a more hydrophobic cell surface, which may be attributed to an increase in surface LPS or changes to LPS structure. From our Western blot analysis, we observed a different banding pattern for OSA (formerly known as B-band) between F0 and F1 (**Figure 2F**). F0 have a uniform distribution of OSA of different lengths while F1 seems to possess predominantly OSA of medium length and low amounts of shorter length OSA while devoid of full length OSA.

TABLE 1 | Antibiotic susceptibility measurement with Etest.

	Minimum inhibitory concentration (μg/mL)	
Antibiotic	F0	F1
Gentamicin	2.000	1.500
Ciprofloxacin	0.094	0.016

DNA Microarray

To study the differences in gene expression between the wild-type F0 and SCV F1 strains, bacterial samples at mid-log

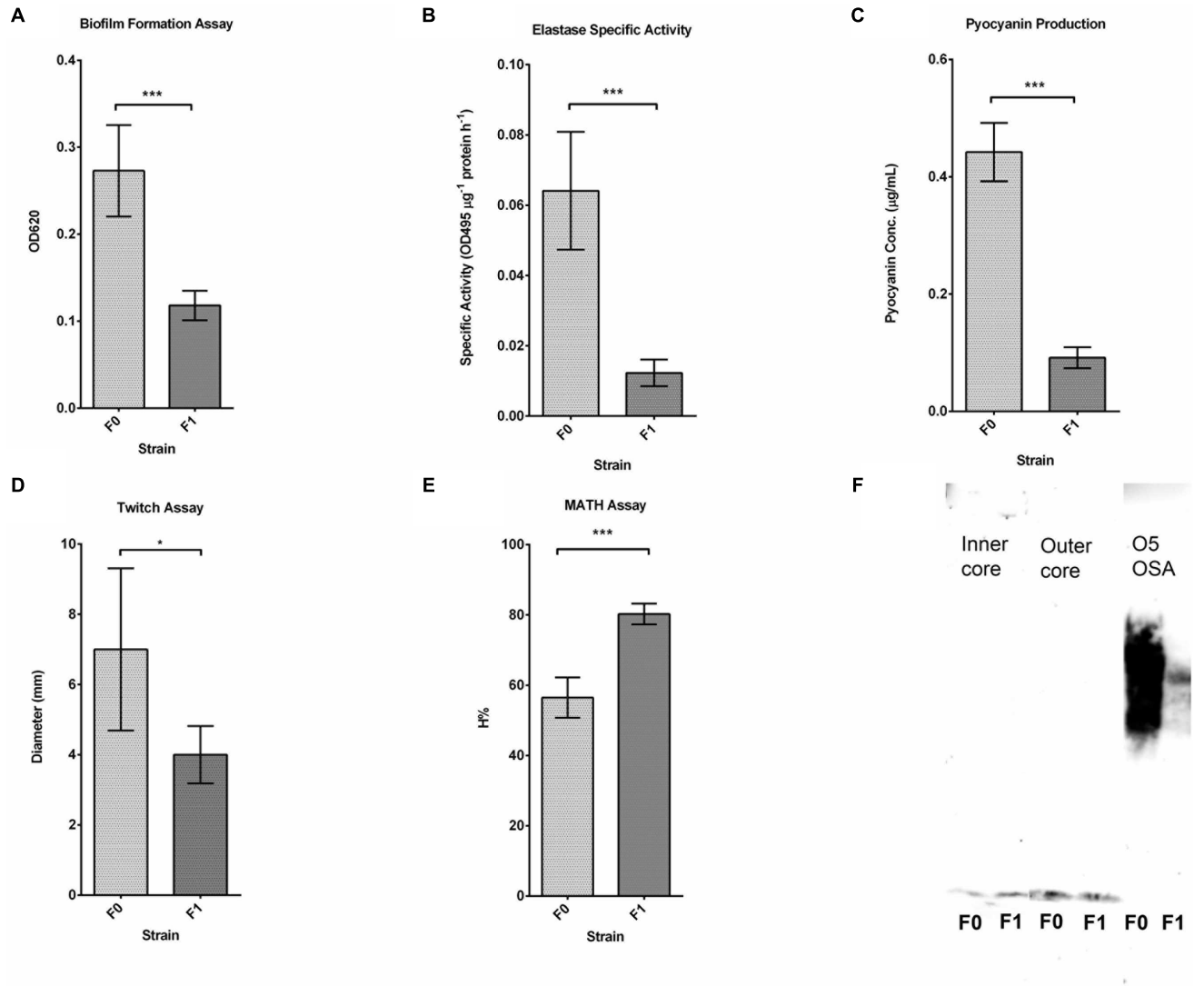

FIGURE 2 | Results for phenotypic assays. (A) Biofilm formation assay of three replicates of F0 and F1 strains; Student's *t*-test. F1 cells have a significantly lower OD260 than F0, indicating fewer attached cells since fewer of them are stained. **(B)** Elastase assay of five replicates of F0 and F1 strains; Student's *t*-test. F1 cells produce significantly lower elastase specific activity than F0. **(C)** Pyocyanin production assay of five replicates of F0 and F1 strains, measured concurrently with elastase activity; Student's *t*-test. F1 cells produce significantly lower amount of pyocyanin than F0. **(D)** Twitch assay for seven replicates of F0 and F1 strains; Student's *t*-test. F1 cells have a significantly lower twitching ability than F0. **(E)** MATH assay of 12 replicates of F0 and F1 strains; Student's *t*-test. F1 cells have a significantly higher H% than F0 cells. **(F)** Western blot analysis of LPS preparations from F0 and F1 strains, tested using antibodies for inner, outer core and OSA. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

growth phase were collected for RNA extraction and cDNA synthesis for DNA microarray transcriptome analysis. After array hybridization and scanning, data was normalized using the Gene Chip (GC) robust multi-array analysis (GCRMA) and probesets were filtered according to their signal intensity and standard deviation (Hackstadt and Hess, 2009). Probesets with low signal (<3.2), constituting noise, and with low standard deviation (<0.25) were removed, yielding 1566 probesets which met the threshold for analysis. This represented an average of 63% of the 5900 open reading frames (ORFs) detected on the array chip. Using a *p*-value criteria of <0.05 and a FC of 1.5 for a DEG, 148 DEGs (**Figures 3A,B**) were identified between F0 and F1. The 148 DEGs represented 2.5% of the total number of ORFs, of which 55 genes were upregulated while 93 were downregulated (Supplementary Tables S1 and S2). Annotating these DEGs according to their PseudoCAP (*Pseudomonas* community annotation project) class functions (**Figure 3C**) revealed that the three largest groups of DEGs belong to metabolism (30%), protein secretion/transport (28%) and hypothetical proteins (27%).

An interesting group of genes that were upregulated in F1 are the *P. aeruginosa* serotype O5 OSA biosynthesis cluster (**Table 2A**) – *wbpI, wbpH, wbpG, hisF2* and *hisF1*. OSA acts as the O-antigen for serotype O5 to which PAO1 belongs. This

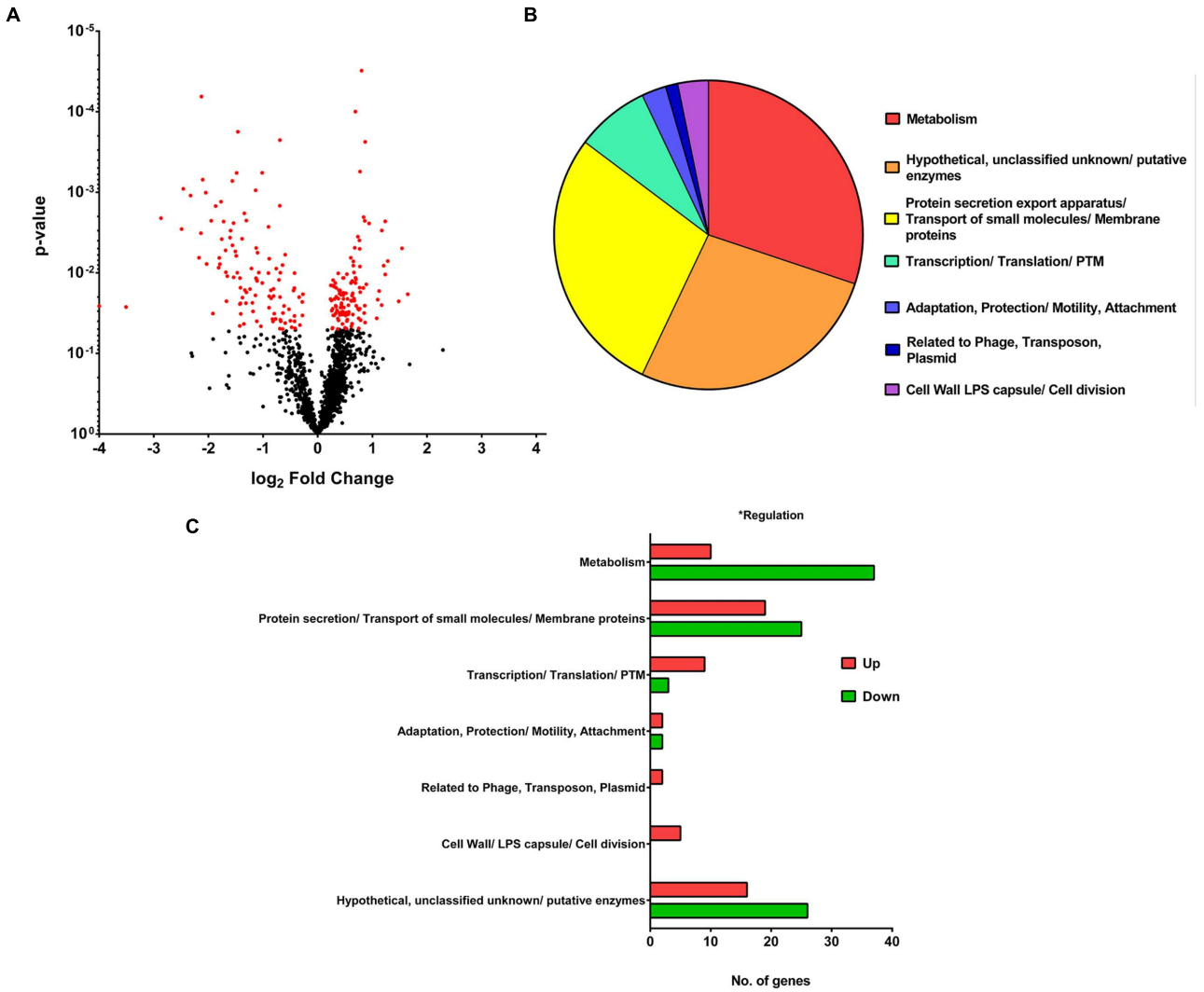

FIGURE 3 | Microarray analysis. (A) Volcano Plot showing the fold change and *p*-value of the 1566 probesets. For a gene to be differentially expressed, it must satisfy the criteria of *p*-value <0.05 and fold change > 1.5 or < −1.5. One hundred and forty-eight differentially expressed genes (DEGs) are shown in red on the plot. **(B)** Pie chart showing PseudoCAP functional classification of DEGs, with metabolism, hypothetical proteins and protein secretion forming the majority. **(C)** Bar graph showing PseudoCAP functional classification of DEGs sorted according to their expression level.

cluster contains 16 genes that are involved in the synthesis of LPS and three other genes that are not (Burrows et al., 1996). This group of genes is also found in serotypes O2, O16, O18, and O20 which have a structurally related O-antigen serogroup. Down-regulated genes include those involved in several amino acid catabolic pathways, such as aromatic amino acid catabolism (**Table 2B**) – *hpd, hmgA, maiA and fahA* and branched chain amino acid (BCAA) catabolism (**Table 2C**) - *bkdA2, bkdB, mmsA, mmsB* and *lpdV* and (**Figures 4A–D**). We proposed that the amino acids may be required as

TABLE 2A | Up-regulated genes involved in *Pseudomonas aeruginosa* serotype O5 B-band LPS biosynthesis.

Locus	Gene	Function	Fold Change	*p*-value
PA3148	*wbpI*	Probable UDP-*N*-acetylglucosamine 2-epimerase	1.8	4.E-02
PA3149	*wbpH*	Probable glycosyltransferase WbpH	2.2	3.E-02
PA3150	*wbpG*	LPS biosynthesis protein WbpG	2.1	2.E-02
PA3151	*hisF2*	Imidazoleglycerol-phosphate synthase, cyclase Subunit	2.4	1.E-02
PA3152	*hisH2*	Glutamine amidotransferase	2.3	3.E-03

TABLE 2B | Down-regulated genes involved in aromatic amino acid catabolism.

Locus	Gene	Function	Fold Change	*p*-value
PA0865	*hpd**	4-hydroxyphenylpyruvate dioxygenase	−4.1	8.E-03
PA0870	*phhC*	Aromatic amino acid aminotransferase	−2.2	1.E-02
PA2007	*maiA**	Maleylacetoacetate isomerase	−2.7	3.E-02
PA2008	*fahA**	Fumarylacetoacetase	−3.2	5.E-03
PA2009	*hmgA**	Homogentisate 1,2-dioxygenase	−2.7	2.E-02

TABLE 2C | Down-regulated genes involved in branched chain amino acid catabolism.

Locus	Gene	Function	Fold Change	*p*-value
PA0744	–	Probable enoyl-CoA hydratase/isomerase	−4.1	1.E-03
PA0745	–	Probable enoyl-CoA hydratase/isomerase	−5.0	1.E-03
PA0747	–	Probable aldehyde dehydrogenase	−1.6	4.E-02
PA1984	–	NAD + dependent aldehyde dehydrogenase	−5.5	9.E-04
PA2000	–	Dehydrocarnitine CoA transferase, subunit B	−1.6	5.E-02
PA2001	*atoB*	Acetyl-CoA acetyltransferase	−1.9	1.E-02
PA2012	*gnyA*	Alpha subunit of geranoyl-CoA carboxylase	−2.6	4.E-02
PA2013	*gnyH*	Gamma-carboxygeranoyl-CoA hydratase	−2.6	2.E-02
PA2014	*gnyB*	Beta subunit of geranoyl-CoA carboxylase	−2.3	2.E-02
PA2247	*bkdA1*	2-oxoisovalerate dehydrogenase (alpha subunit)	−2.7	1.E-02
PA2248	*bkdA2**	2-oxoisovalerate dehydrogenase (beta subunit)	−3.4	4.E-03
PA2249	*bkdB**	Branched-chain alpha-keto acid dehydrogenase (lipoamide component)	−3.4	1.E-03
PA2250	*lpdV**	Lipoamide dehydrogenase-Val	−3.0	4.E-03
PA2553	–	Probable acyl-CoA thiolase	−5.6	3.E-03
PA2554	–	Probable short-chain dehydrogenase	−2.2	1.E-02
PA3569	*mmsB**	3-hydroxyisobutyrate dehydrogenase	−4.3	7.E-04
PA3570	*mmsA**	Methylmalonate-semialdehyde dehydrogenase	−4.5	6.E-03

**Denotes genes that were validated with qPCR.*

substrates for other pathways hence they are from being catabolized.

Identifying Unique F1 Single Nucleotide Variations by Whole Genome Sequencing

Since the SCV phenotype of F1 is stable, whole genome sequencing to identify single nucleotide variations (SNVs) was performed to elucidate the underlying genomic changes in F1 compared to wild-type F0. From the SNV list, we needed to identify SNVs that were present only in F1 but absent in F0. We pooled all the SNVs found in F1 and F0 separately and compared the SNVs. Using this approach, 64 SNVs were identified that were unique only to F1 (**Table 3**). These SNVs were located in the region from PA0717-PA0729 (**Figure 5A**) with the PseudoCAP class function of "related to phage, transposon and plasmid." Three SNVs were from intergenic regions while 61 were from coding regions. Of the 61 SNVs, 18 were transversions while the rest was transitions. Furthermore, 11 of the 61 SNVs in the coding region, 11 SNVs correspond to non-synonymous mutations while the remainder 50 SNVs corresponds to synonymous mutations (**Figures 5B,C**). It was observed that transversions and non-synoymous SNVs tend to congregate nearer to the 5′ end of the region (**Figure 5B**).

It is puzzling that F1 carried so many synonymous mutations that led to no change in amino acids in the coded proteins. Synonymous mutations may affect the regulation of protein expression at the stage of translation whereby certain codons are much preferred to code for a particular amino acid. This form of codon usage bias has been extensively described in *Escherichia coli* (Sharp et al., 1988; Pek et al., 2015). Studying codon bias in *P. aeruginosa*, Grocock and Sharp (2002) concluded that a weak codon bias is present in *P. aeruginosa* and calculated relative synonymous codon usage (RSCU) values for each codon. Using their RSCU values, we investigated whether there is a trend of F1 using preferred codons, as specified by its 50 synonymous mutations, to code for particular amino acids. Synonymous SNVs were split into two groups based on whether a less or more efficient codon for a particular amino acid was used. We identified 29 SNVs where a preferred codon was used and 22 SNVs where a less-preferred codon was used. For each of these groups, the proportion for each class of amino acid side chain that was found in the group was counted. We deduced that aromatic and BCAAs were preferentially coded using more efficient codons (**Figure 5D**). An overview of these findings is presented in **Figure 5D** which displays the type of SNV at each position. To address whether such SNVs are common among all SCVs after infecting *P. aeruginosa* with PB1 phage, we isolated four additional SCVs derived from PB1 infection of F0 and sequenced their genomes. 55 of the 61 SNVs that were identified in F1 were also present in these 4 SCVs (**Figure 5E**).

TABLE 3 | Single nucleotide variations unique to F1 strain compared to the wild-type.

Locus (No. SNVs)	Position	Ref base	Genotype call	Ref amino acid	Amino acid call	PseudoCAP class function	
PA0719	789941[a]	C	G	Ser	Arg	Hypothetical	Related to phage, transposon, or plasmid
(3)	789960	A	C	Arg	Arg		
	789985[a]	C	A	Ser	Tyr		
PA0720	790228	T	C	Tyr	Tyr	DNA replication, recombination, modification and repair	Related to phage, transposon, or plasmid
(11)	790267	A	G	Gln	Gln		
	790312	A	C	Gly	Gly		
	790360	C	T	Ile	Ile		
	790408[b]	T	C	Arg	Arg		
	790411[b]	G	C	Pro	Pro		
	790447[b]	G	A	Gln	Gln		
	790453[b]	A	T	Leu	Leu		
	790521[a,b]	T	C	Val	Ala		
	790540[b]	C	T	Arg	Arg		
	790580[a,b]	A	T	Thr	Ser		
Intergenic	790604[b]	C	G				
PA0724	791486	G	A	Ser	Ser		Related to phage, transposon, or plasmid
(4)	791518[a]	C	A	Ala	Asp		
	791626[a]	G	C	Gly	Ala		
	791651	C	T	Gly	Gly		
PA0726	793023[b]	C	G	Pro	Pro	Hypothetical	Related to phage, transposon, or plasmid
(11)	793026[b]	T	C	Asn	Asn		
	793056	G	A	Gln	Gln		
	793371	C	G	Leu	Leu		
	793374	C	T	Asp	Asp		
	793389	T	C	His	His		
	793410	T	C	Ile	Ile		
	793411[a]	G	A	Val	Ile		
	793432[a]	T	G	Ser	Ala		
	793437	C	T	Tyr	Tyr		
	793449	T	C	Asp	Asp		
PA0727	794872	G	A	Glu	Glu	Hypothetical	Related to phage, transposon, or plasmid
(19)	794875	A	G	Val	Val		
	795082	G	T	Arg	Arg		
	795289	C	G	Val	Val		
	795307	C	T	Arg	Arg		
	795331	G	A	Gln	Gln		
	795349	C	T	Arg	Arg		
	795355	T	C	Ala	Ala		
	795361	A	G	Gly	Gly		
	795391	G	C	Gly	Gly		
	795394	T	C	Leu	Leu		
	795403	C	T	His	His		
	795457	G	A	Thr	Thr		
	795499	C	T	Ala	Ala		
	795553	T	C	Tyr	Tyr		
	795574	T	C	Phe	Phe		
	795583	T	C	Arg	Arg		
	795598	T	C	Phe	Phe		
	795652	T	G	Ala	Ala		
PA0728	795867	G	A	Lys	Lys	Putative enzymes	Related to phage, transposon, or plasmid
(10)	796015	T	C	Leu	Leu		
	796035	C	T	Cys	Cys		
	796125	A	G	Arg	Arg		
	796161	C	T	Asn	Asn		
	796203	C	T	Arg	Arg		
	796273	T	C	Leu	Leu		
	796443	T	C	Phe	Phe		
	796446	C	T	Ala	Ala		
	796452	T	C	Thr	Thr		

(continued)

TABLE 3 | Continued

Locus (No. SNVs)	Position	Ref base	Genotype call	Ref amino acid	Amino acid call	PseudoCAP class function
Intergenic	796789	T	G			
	797040	T	C			
PA0729	797516	T	C	Val	Ala	Hypothetical
(3)	797533	T	C	Leu	Leu	
	797550	C	T	Ser	Ser	

[a]*Denotes a non-synonymous SNV.* [b]*Denotes SNVs that are not found in the other 4 SCVs isolated.*

DISCUSSION

In this study, SCV were isolated from the surviving population of wild-type *P. aeruginosa* PAO1 after 24 h of exposure to PB1 phage. The SCV isolates were found to be resistant to further PB1 infection and displayed several phenotypic changes. Compared to the wild-type F0, the PB1 phage-resistant F1 are less competent at attaching to surfaces in the biofilm formation assay (**Figure 2A**), produces lower amounts of the virulence factors elastase and pyocyanin (**Figures 2B,C**), and do not twitch as much (**Figure 2D**). Since both attachment and twitching are important processes in the formation of biofilms (O'Toole and Kolter, 1998; Ramsey and Whiteley, 2004), F0 would be expected to have greater biofilm formation than F1. Consistent with reports on bacterial SCVs, reduced twitching ability is also associated with smaller colonies on agar (Proctor et al., 2006). The surface of F1 is also more hydrophobic than F0 (**Figure 2E**), allowing F1 cells to aggregate more easily through hydrophobic

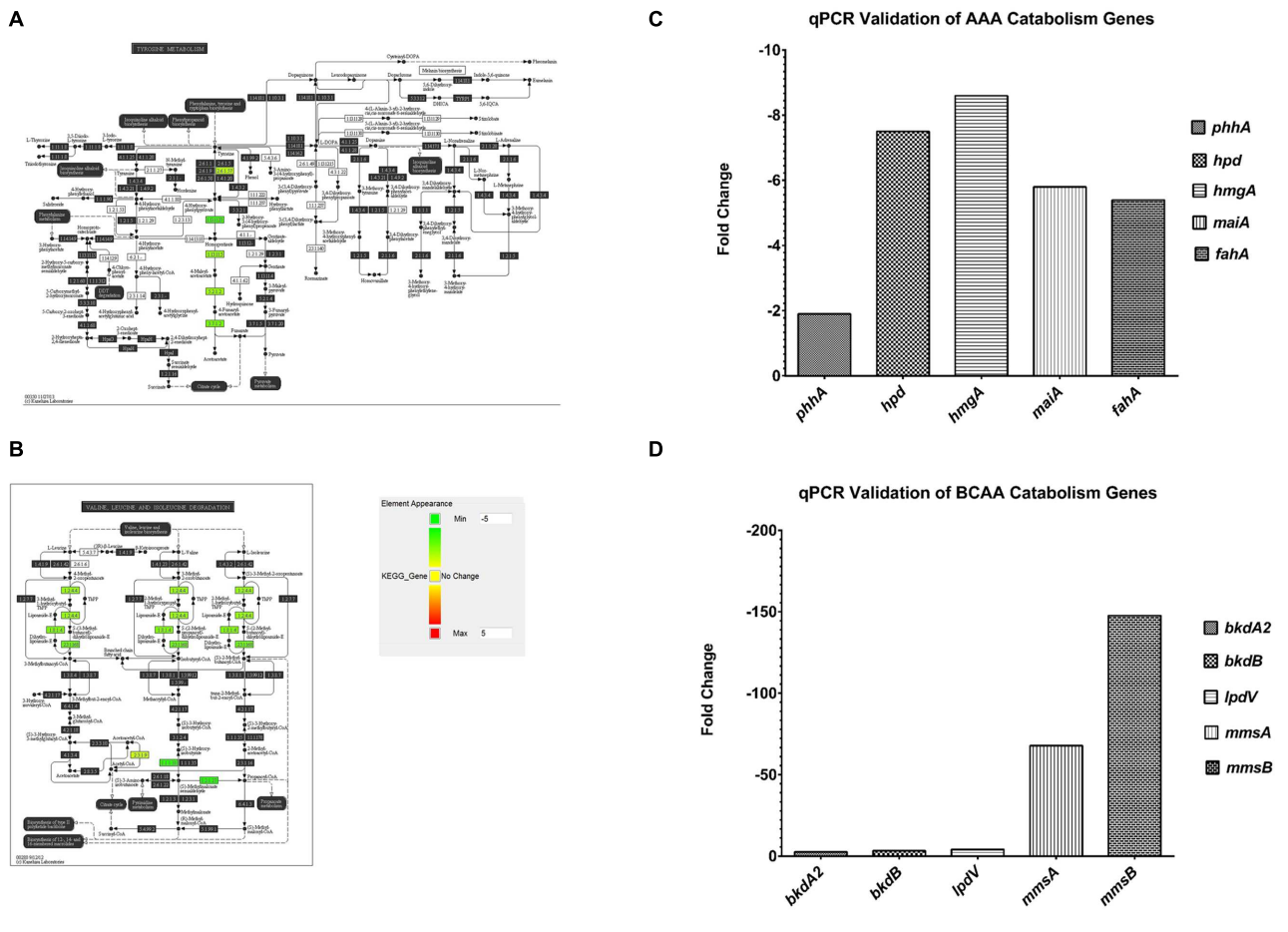

FIGURE 4 | Pathway analysis of microarray results. (A) Pathway analysis- 5 genes, *phhA*, *hpd, hmgA, maiA* and *fahA* from the aromatic amino acid catabolism pathway were downregulated (green). **(B)** Pathway analysis- 5 genes, *bkdA2*, *bkdB, lpdV, mmsA* and *mmsB* from the branched chain amino acid catabolism pathway were downregulated (green). **(C)** A representative qPCR validation of *phhA*, *hpd, hmgA, maiA* and *fahA*, normalized to *rpoD*. **(D)** A representative qPCR validation of *bkdA2*, *bkdB, lpdV, mmsA* and *mmsB*, normalized to *rpoD*.

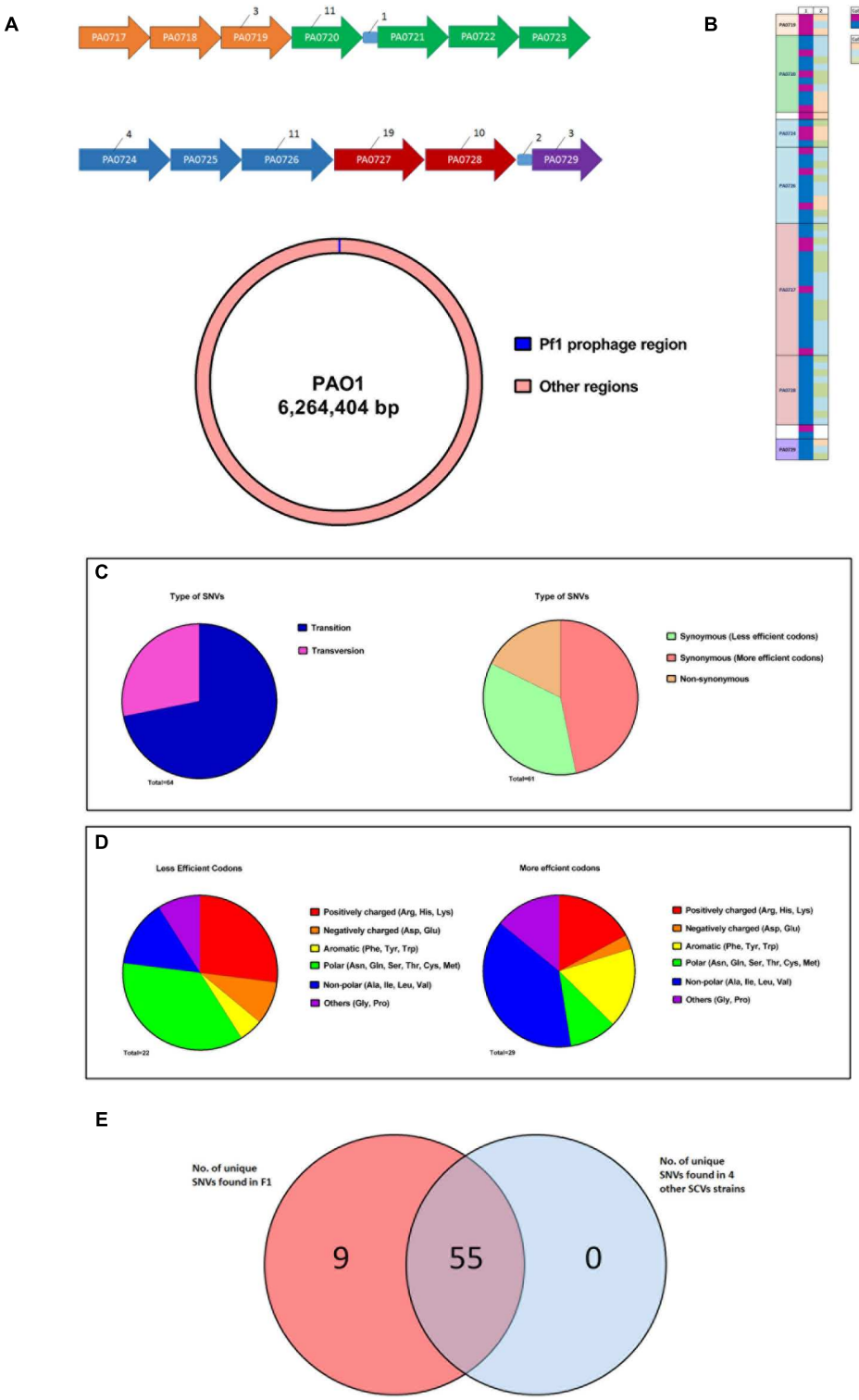

FIGURE 5 | Whole genome sequencing of F1 to identify unique SNVs. (A) Figure showing the distribution of unique F1 strains SNV across the Pf1 prophage regions. All intergenic regions except those containing SNVs were removed for clarity. Numerical values indicate the number of SNVs found in each gene. Genes of the same color belongs to the same operon. **(B)** A schematic showing the size of Pf1 prophage region (~8 kb) in relation the size of the PAO1 genome (~6 Mb) **(C)** Synoymous SNVs were split into two groups based on whether a less or more efficient codon for a particular amino acid was used in F1 and the type of amino acid side chains were shown in the pie charts. Aromatic and branched chain amino acids (yellow and blue regions respectively) were preferentially using a more efficient codon. **(D)** Diagram showing the attributes of the SNVs found in each locus. **(E)** Venn diagram showing the distribution of these 64 SNVs across 4 other SNV isolated from F0. 55 of these SNVs are common to all five strains.

interactions and hence able to form smaller-sized colonies than F0, similar to clinical variants reported by Kirisits et al. (2005). The production of virulence factors, pyocyanin and elastase (**Figures 2D,E**) are co-regulated through the *las* quorum sensing system (Pearson et al., 1997; Glessner et al., 1999; Sauer et al., 2002). The lower expression of both factors in F1 may indicate that the *las* system is deficient in F1 strains.

From the microarray data, several genes from *P. aeruginosa* serotype O5 OSA biosynthesis cluster were found to be upregulated. OSA is made up of repeating units of sugars which acts as the serotype specific O antigen (Burrows et al., 1996). The structure of OSA varies among the different *P. aeruginosa* serotypes and PAO1 strain belongs to the O5 serotype. OSA has a varying chain length based on the number of repeating units (Rocchetta and Lam, 1997). The chain length of the OSA is regulated by *wzz* (Daniels et al., 2002). The expression pattern of several OSA genes was upregulated in the F1 over the F0 strain (**Table 2A**), indicating alteration of the structure of OSA in F1. These observations were supported through Western blot analysis of LPS preparations using antibodies specific to the inner, outer core and OSA. The results showed a markedly different distribution of OSA banding pattern between the two strains. We observed the absence of high molecular weight OSA and lower amounts of low molecular weight OSA in the F1 over the F0 strain. This could have contribute to the lower surface hydrophobicity observed in the MATH assay which suggest change in structure of LPS. In the study by Garbe et al. (2010), they concluded that the receptor for the PB1-like phage JG024 is a component of the core LPS. Changes to the OSA could play a role in preventing binding of phage particles and initializing the infection process. From these results, we can postulate that differences in surface properties between the strains may prevent PB1 phage from binding to LPS receptors on F1 surface.

Infection by PB1 phages could have induced a stress response in the *P. aeruginosa* that caused the bacteria to accumulate spontaneous mutations. Phage-driven changes in phenotype have been previously shown for PP7, a ssRNA, pilus-binding phage. Similar to PB1, PP7 induces SCVs in *P. aeruginosa* (Brockhurst et al., 2005). Under stressful conditions, the SOS response in bacteria is activated, thus allowing the bacteria to undergo mutations on a genome wide scale (McKenzie et al., 2000). Certain regions of the genome tend to be hypermutable, and indeed, our sequencing results show that SNVs were predominantly found in the Pf1 prophage region, located at positions PA0719–PA0729 of the *P. aeruginosa* genome. This region originated from a horizontal gene transfer event from Pf1 filamentous lysogenic phages, to ensure that their genetic material is preserved in the bacteria, and has an unusually low GC-content when compared to the rest of the genome. In a study by Wei et al. (2011), where *P. aeruginosa* SCV was generated by exposing wild-type bacteria to high concentrations of gentamicin. One key difference, however, is that the SCV in our study showed an increased susceptibility to ciprofloxacin, while theirs showed an increased resistance to the antibiotic. They also discovered a silent mutation in the elongation factor *tufA* in revertant cells that could be the cause of their increased growth rate. In a similar fashion, the silent mutations found in the Pf1 region in this study may contribute to codon bias and resulted in one or more of the phenotypes observed. Filamentous phage have been known to exhibit high frequency of mutations which could explain the presence of SNVs found in the Pf1 region (Kuo et al., 2000). Previous studies have shown that cells can acquire a superinfective phenotype, capable of killing cells in *P. aeruginosa* biofilms, through mutations in the Pf1 region, (Webb et al., 2003). Similarly, Pf1 prophage might be involved in the selection of F1 when F0 was first exposed to PB1 phage. This implicates a possible role in the Pf1 prophage region of *P. aeruginosa* for the generation of the SCV phenotype.

AUTHOR CONTRIBUTIONS

WL designed and performed the experiments, contributed to the results interpretation, manuscript writing and approval of the final version for publication; KP performed the experiments, contributed to the results interpretation and approval of the final version for publications; AT, SL and DO contributed to the results interpretation and approval of the final version for publication.

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Characterization of Five Podoviridae Phages Infecting *Citrobacter freundii*

Sana Hamdi[1,2], Geneviève M. Rousseau[3], Simon J. Labrie[3], Rim S. Kourda[1,2], Denise M. Tremblay[3], Sylvain Moineau[3] and Karim B. Slama[1,2]**

[1] *Laboratoire des Microorganismes et Biomolécules Actives, Faculté des Sciences de Tunis, Université de Tunis-El Manar, Tunis, Tunisie,* [2] *Département de Biotechnologie, Institut Supérieur des Sciences Biologiques Appliquées de Tunis, Université de Tunis El-Manar, Tunis, Tunisie,* [3] *Département de Biochimie, de Microbiologie, et de Bioinformatique and PROTEO, Faculté des Sciences et de Génie, Félix d'Hérelle Reference Center for Bacterial Viruses, and GREB, Faculté de Médecine Dentaire, Université Laval, Québec City, QC, Canada*

Citrobacter freundii causes opportunistic infections in humans and animals, which are becoming difficult to treat due to increased antibiotic resistance. The aim of this study was to explore phages as potential antimicrobial agents against this opportunistic pathogen. We isolated and characterized five new virulent phages, SH1, SH2, SH3, SH4, and SH5 from sewage samples in Tunisia. Morphological and genomic analyses revealed that the five *C. freundii* phages belong to the *Caudovirales* order, *Podoviridae* family, and *Autographivirinae* subfamily. Their linear double-stranded DNA genomes range from 39,158 to 39,832 bp and are terminally redundant with direct repeats between 183 and 242 bp. The five genomes share the same organization as coliphage T7. Based on genomic comparisons and on the phylogeny of the DNA polymerases, we assigned the five phages to the *T7virus* genus but separated them into two different groups. Phages SH1 and SH2 are very similar to previously characterized phages phiYeO3-12 and phiSG-JL2, infecting, respectively, *Yersinia enterocolitica* and *Salmonella enterica*, as well as sharing more than 80% identity with most genes of coliphage T7. Phages SH3, SH4, and SH5 are very similar to phages K1F and Dev2, infecting, respectively, *Escherichia coli* and *Cronobacter turicensis*. Several structural proteins of phages SH1, SH3, and SH4 were detected by mass spectrometry. The five phages were also stable from pH 5 to 10. No genes coding for known virulence factors or integrases were found, suggesting that the five isolated phages could be good candidates for therapeutic applications to prevent or treat *C. freundii* infections. In addition, this study increases our knowledge about the evolutionary relationships within the *T7virus* genus.

Keywords: *Citrobacter freundii*, pathogen, phages, virulent, *T7virus*, therapeutic applications

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Peter Mullany,
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Evelien M. Adriaenssens,
University of Pretoria, South Africa
Shan Goh,
Royal Veterinary College, UK

****Correspondence:***
Sylvain Moineau
sylvain.moineau@bcm.ulaval.ca;
Karim B. Slama
karim.benslama@fst.rnu.tn

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INTRODUCTION

Members of the Gram-negative *Enterobacteriaceae* have caused significant diseases throughout human history. They are responsible for many human infections in the intestine, urinary tract, bloodstream, and wounds (Abbott, 2011; Shanks et al., 2012). The genus *Citrobacter* belongs to this bacterial family, although it was originally classified within the genus *Salmonella* due to biochemical and serological similarities (Harhoff, 1949; Ewing and Davis, 1972). *Citrobacter freundii* is the type species of this genus, with a genome size of ∼5 Mb and a G+C content of 50 to 52%

(Kumar et al., 2013; Kimura et al., 2014). *C. freundii* is commonly found in soil, water, foods, and the intestinal tracts of animals and humans (Drelichman and Band, 1985). Some strains of *C. freundii* can also cause opportunistic infections in humans and animals, which are becoming more difficult to treat due to increased antibiotic resistance. As such, *C. freundii* infections have become a public health concern (Samonis et al., 2008; Antonelli et al., 2015; Campos et al., 2015) and alternatives or adjuncts to antibiotic treatment are required.

In this context, lytic/virulent phages are being re-investigated as potential antimicrobial agents to either combat bacterial diseases or to stop the dissemination of multi-resistant bacteria. The potential of phages to control or treat bacterial diseases has been previously demonstrated (Smith and Huggins, 1982; Slopek et al., 1983). However, their use was mostly abandoned for several well-documented reasons including the inability to purify phage preparations from bacterial components, the lack of understanding of basic phage biology, the inability to differentiate temperate from lytic phages, narrow host ranges, the development of phage-resistant bacterial mutants, and the inherent difficulties of patenting phages and their use. It is believed that progress has been made to overcome most, if not all, these difficulties (Carlton, 1999; Loc-Carrillo and Abedon, 2011).

Several phages infecting various strains of *C. freundii* have been recently characterized. Six of them belong to the *Myoviridae* family [double-stranded DNA genome (dsDNA), contractile tail] and were isolated from water samples in Texas. Their genomic characterization indicated that three of these phages (Moon, Miller, Merlin) are related to the *T4virus* genus (Edwards et al., 2015; Hwang et al., 2015; LeSage et al., 2015) while the other three (Mordin, Michonne, Moogle) are related to the *Felixo1virus* genus (Bernal et al., 2015; Guan et al., 2015; Nguyen et al., 2015). The complete genomic sequence of the *C. freundii* phage Stevie is also available (Shaw et al., 2015). This *Siphoviridae* phage (dsDNA, noncontractile tail), which was isolated from a dirt sample in Texas, is related to the *T1virus* genus. Phages of the *Podoviridae* family (dsDNA, short tail) can also infect *C. freundii* strains as the podophage LK1 was isolated from sewage and its genome size was estimated to be 20–23 kb (Chaudhry et al., 2014). The podophage phiCFP-1 was isolated from sewage in China and classified as a *T7virus* with a genome of 38,625 bp with 43 *orfs* and direct terminal repeats of 229 bp (Zhao et al., 2015).

Phages belonging to the *T7virus* genus are particularly interesting for therapeutic applications as they are usually easy to culture and have a short lytic cycle. They also have smaller genomes and a conserved organization, which facilitates their in-depth analysis. Their genomes can be divided into three transcriptional regions including early-, middle-, and late-expressed genes (Scholl and Merril, 2005; Zhu et al., 2010). As for the prototype coliphage T7, the genes of these phages can be transcribed due to an efficient phage-encoded RNA polymerase that specifically recognizes a set of conserved promoters dispersed throughout the phage genome (Chen and Schneider, 2005; Huang et al., 2012).

Here, we describe five lytic *Podoviridae* phages infecting *C. freundii* isolated from sewage samples in Tunisia. Their analyses showed that they belong to the *Autographivirinae* subfamily and they share similarities with phages infecting other *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial Strains, Phage Isolation, and Culture Conditions

Five bacterial isolates were obtained by plating Tunisian wastewater samples on *Salmonella-Shigella* agar (Biokar) and incubating the plates for 24 h at 37°C. The species of each bacterial isolate was determined by 16S rRNA sequencing and API 20 E strip (BioMérieux). *C. freundii* strains were genotyped using multi-locus sequence typing (MLST) of seven housekeeping genes (*aspC*, *clpX*, *fadD*, *mdh*, *arcA*, *dnaG*, and *lysP*) as described previously (Bai et al., 2012). The allelic profile and sequence type (ST) of each strain was identified using the MLST database website (http://pubmlst.org/cfreundii/). Evolutionary analyses were conducted with MEGA7 (Kumar et al., 2016). The neighbor-joining phylogenetic tree (Saitou and Nei, 1987) of the five strains was generated from the concatenated sequences of the seven loci. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site.

Two *C. freundii* isolates were used as hosts for phage isolation. Water samples were obtained from four different areas in Tunis (**Table 1**). One millilitre of the filtered water samples was mixed with 1 ml of an overnight bacterial culture in 3 ml of Brain Heart Infusion broth (BHI) (Biokar or BD). After incubation for 24 h at 37°C, the mixtures were centrifuged and 4 μl of each filtered-supernatant was spotted on a fresh bacterial lawn. After incubation at 37°C for 24 h, phage lysis zones were picked with a sterile truncated tip and amplified in the presence of their respective host in BHI for 24 h at 37°C. Then, the mixtures were centrifuged and the supernatants filtered. Isolated plaques were obtained using the double-layer agar method and picked with a sterile truncated tip. This step was repeated three times to ensure phage purity. Phages and bacterial strains were deposited at the Félix d'Hérelle Reference Center for Bacterial Viruses of the Université Laval (www.phage.ulaval.ca) under the following names: phages SH1 (HER 516), SH2 (HER 517), SH3 (HER 518), SH4 (HER 519), and SH5 (HER 520) as well as *C. freundii* strains CF3 (HER 1518) and CF5 (HER 1516).

TABLE 1 | Origins of phages and their host strains.

Phage	Origins of phages	Host strain	Origins of strains
SH1	Wadi of Khaznadar	CF5	Entry water treatment plant of Menzah1
SH2	Wastewater from Mellassine	CF5	
SH3	Office of national sanitation of Ksar Said	CF3	Entry water treatment plant of Gammarth
SH4	Wastewater from Mellassine	CF3	
SH5	Wadi of Ezzouhour city	CF3	

Microbiological Assays

The host range of the five phages was determined by spotting 4 μl of various serial dilutions (10^0 to 10^{-7}) of a phage lysates on BHI soft agar (0.75% agar) containing one bacterial strain. After overnight incubation at 37°C, plates were examined for the presence of isolated plaques in the spotted areas, which indicated a full phage lytic cycle on the host. The host range was tested on 5 *C. freundii* (this study), one *Cronobacter turicensis* (290708/07) and 25 bacterial strains available at the Félix d'Hérelle Reference Center for Bacterial Viruses of the Université Laval: 10 *Escherichia coli* (HER1024, HER1040, HER1144, HER1255, HER1462, HER1155, HER1290, HER1022, HER1213, and HER1445), two *Shigella dysenteriae* (HER1020 and HER1031), one *Shigella sonnei* (HER1043), two *Salmonella* Paratyphi (HER1045, HER1220), one *Salmonella* Typhi (HER1038), two *Salmonella* Typhimurium (HER1023, HER1095), two *Salmonella* Newport (HER1185 and HER1019), one *Salmonella* Heidelberg (HER1428), one *Salmonella* Senftenberg (HER1397), and 3 *Yersinia enterocolitica* (HER1249, HER1071, HER1072). Phage susceptibility to pH (2 to 10) was also determined in BHI broth with the pH adjusted using hydrochloric acid (HCl) or sodium hydroxide (NaOH). One hundred microlitre of each phage lysate at $>10^9$PFU/ml were mixed with 900 μl of media for each pH condition and incubated at 37°C for 60 min. Phage titer was then determined using the double-layer agar method.

Electron Microscopy

Phages were prepared and observed as described previously (Fortier and Moineau, 2007). The reported dimensions are the means of at least ten virions stained with uranyl acetate (2%).

Phage Structural Proteins

Phages were precipitated from lysates (1L) with 10% polyethylene glycol (PEG) 8000 and 2922g of sodium chloride then concentrated using a discontinuous CsCl gradient followed by a continuous CsCl gradient, as described previously (Chibani Azaïez et al., 1998; Sambrook and Russel, 2001). A purified phage sample was sent directly for structural protein identification by liquid chromatography/tandem mass spectrometry (LC-MS/MS) at the Plateforme Protéomique, Centre de Génomique de Québec (Université Laval). A custom database was generated using the putative predicted proteins. Results were analyzed using Scaffold Proteome software version 4.4.5.

Genome Sequencing and Bioinformatics Analyses

Phage DNA was extracted from high titer phage lysates using a Plasmid Maxi Kit (Qiagen) with modifications described elsewhere (Deveau et al., 2002). Phage DNA was prepared for sequencing using the Nextera XT DNA library preparation kit (Illumina) according to the manufacturer's instructions. The libraries were then sequenced on a MiSeq system using a MiSeq reagent kit v2 (Illumina, 500 cycles). *De novo* assembly was performed with Ray assembler version 2.2.0 using *k*-mer sizes of 21, 51, 96, 31, and 51 and we obtained mean coverage depths for each single phage contig of 2717, 1643, 3804, 134, and 2431 for SH1, SH2, SH3, SH4, and SH5, respectively. Coverage was calculated with Samtools. Open reading frames (ORFs) were identified using ORF Finder (Rombel et al., 2002) and GeneMark (Lukashin and Borodovsky, 1998) then confirmed by visual inspection for the presence of a Shine-Dalgarno sequence close to a start codon (AUG, UUG or GUG) using BioEdit 7.2.0 (Hall, 1999). ORFs were considered if they contained at least 30 amino acids (aa). Similarities with known proteins were searched with BLAST. Hits were considered when the *E*-value was lower than 10^{-3}. The percentage of identity between proteins was calculated by dividing the number of identical residues by the size of the smallest protein. The theoretical molecular weight (MW) and isoelectric point (pI) of the ORFs were calculated using the Compute pI/MW tool (http://web.expasy.org/compute_pi/).

Determination of Genome Ends

To confirm the direct terminal repeats, primers adjacent to the predicted terminal ends were designed using Primer-BLAST at NCBI. The putative ends were established by aligning the genome termini with similar phage genomes using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The primers were used to sequence directly from the phage DNA at the sequencing and genotyping platform of the Université Laval using the ABI data 3730XL DNA analyzer. The primers used are described in **Table 2**. Terminal repeat sequences were determined using Staden software (version 1.7.0) (Staden, 1996).

DNA Polymerase Phylogeny

The DNA polymerase sequence dataset used for phylogeny included phage proteins from different families and genera (Labrie et al., 2013). The sequences were aligned using MAFFT with the E-INS-i parameter (Katoh and Standley, 2013). The alignment was then processed to generate the tree as previously described (Mercanti et al., 2015). Briefly, the best amino-acid substitution model implemented in PhyML 3.0 to calculate the best tree was predicted with ProtTest 3.2 (Darriba et al., 2011). The Shimodaira-Hasegawa-like procedure was used to determine the branch support values (Shimodaira, 2002). Finally, Newick utility package (Junier and Zdobnov, 2010) and ITOL (Letunic and Bork, 2011) were used to render the tree.

Nucleotide Sequence Accession Numbers

The annotated phage genomic sequences were deposited in GenBank under the numbers KU687347 (SH1), KU687348 (SH2), KU687349 (SH3), KU687350 (SH4), KU687351 (SH5).

TABLE 2 | Primers used to determine the terminal repeats.

Phage	Forward primer (5′-3′)	Reverse primer (5′-3′)
SH1	GCCTCACTGTTCCGTCATTT	CAACTGAAAGGAGGTGGCTC
SH2	TGTCTCAGGGAGTGGCTTTA	GCTCAATGTTACGCTTGCTG
SH3	GCCCTACCCCAGTCTATCAT	CTATCCCTACGCCATCTTGC
SH4/SH5	CTGCTGTTCTACTTGCTGCT	GCTATGGTCCCTGACTGCTA

RESULTS

Isolation of Bacteria and Phages

Five bacterial strains were isolated from different wastewater samples. Gram staining showed Gram negative bacilli. Sequencing of 16S rRNA and API 20E strip identification revealed that they belong to the *C. freundii* species. MLST analyses showed that the five strains also belong to different genotypes, CF5 belong to ST19 and the four other strains belong to four novel and different ST. Phylogenetic analyses (**Figure 1**) revealed that CF3, CF4, and CF7 belonged to a different branch from CF5 and CF8. Two *C. freundii* isolates (CF3 and CF5) were selected from each branch and used as host organisms to isolate phages.

A total of five virulent phages, SH1, SH2, SH3, SH4, and SH5, were isolated from four sewage samples (**Table 1**). For phages SH1 and SH2, plaques of 2 mm in diameter appeared after only 3 h of incubation at 37°C and the plaques became larger with diameters ranging from 4 to 6 mm after overnight incubation, as shown in **Figure 2**. Phage SH3 produced smaller plaques of 1 mm in diameter while phages SH4 and SH5 produced plaques of about 3 mm in diameter.

The host range of the five phages was determined using the 31 Gram-negative bacterial strains described in the Materials and Methods section. Phages SH1 and SH2 were able to lyse their host strain, *C. freundii* CF5, and *S.* Typhi HER1038. Phage SH3 was able to lyse its host strain, *C. freundii* CF3 and *C. freundii* CF4. Phages SH4 and SH5 lysed their host strain, *C. freundii* CF3, as well as *C. freundii* CF4 and *C. turicensis* 290708/7.

Sensitivity to pH

The five phages were tested for their susceptibility to different pH conditions. They were exposed to pHs ranging from 2 to 10 for 1 h at 37°C. All phages were completely inactivated when exposed to pH 2 and pH 3. A 10-fold reduction in phage titer was also noticed at pH 4. All phage suspensions were stable from pH 5 to pH 10.

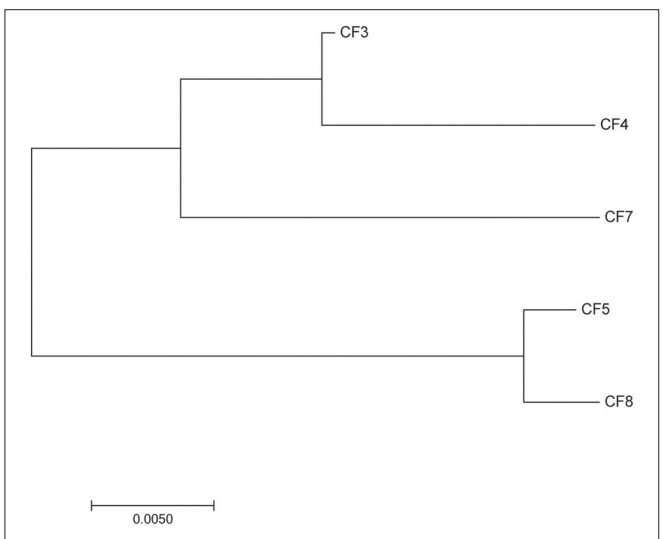

FIGURE 1 | Neighbor-joining phylogenetic tree of the five strains of *Citrobacter freundii*.

Morphological Characteristics

Negatively stained purified phages were observed with an electron microscope and all five possessed an icosahedral capsid and small non-contractile tail (**Figure 3**, **Table 3**). However, the tips of the tails differed which led us to divide them into two morphological groups. The first group included phages SH1 and SH2, which had a narrower base plate compared to the second group, which included phages SH3, SH4, and SH5 (**Figure 3**). Nonetheless, their overall morphology allowed us to classify the five phages into the *Caudovirales* order and the *Podoviridae* family.

Genomic Characteristics

The double-stranded DNA of the five phages was extracted and sequenced. The genome size of these phages ranged from 39,158 to 39,832 bp, which was similar to that of coliphage T7 (39,936 bp) (**Table 3**). The GC contents of the phage genomes were similar to that of their *C. freundii* hosts, 50 to 51% (Frederiksen, 2015). After genome alignments with similar phages, primers adjacent to the predicted terminal ends were used to directly sequence the phage genomic DNA. As expected, the sequencing signal dropped at the end of the genome (**Figure 4**) and this was used to determine the position of the terminal ends and their sequences. The last adenine at the end of the repeated sequences was not considered because it is added by the polymerase (Clark, 1988; Garneau et al., 2010). Our analyses revealed that the five *Podoviridae* phage (podophage) genomes contained direct terminal repeats at both ends (**Table 3**). The length of the direct terminal repeats of phages SH1 (230 bp) and SH2 (242 bp) were similar to that of *Yersinia* phage phiYeO3-12 (232 bp; Pajunen et al., 2001), *Salmonella* phage phiSG-JL2 (230 bp; Kwon et al., 2008), and *Citrobacter* phage phiCFP-1 (229 bp; Zhao et al., 2015). Terminal repeat lengths of SH3 (183 bp), SH4 (190 bp), and SH5 (190 bp) were close to the length of coliphage K1F (179 bp; Scholl and Merril, 2005).

Genome Organization

Analyses of the predicted *orfs* in the genomes of the five newly isolated podophages revealed that they all have the same transcriptional orientation and use only ATG as an initiation codon (**Tables 4**, **5**). Comparative genome analyses also indicated that these phages were affiliated with the *Autographivirinae* subfamily and the *T7virus* genus. Similar to the morphological groupings, we could also divide the five phage genomes into subgroups (**Figure 5**). The first group included phages SH1 and SH2, which had high identity (80%) to genes of *Yersinia* phage phiYeO3-12 as well as coliphages T7 and T3. The second phage group (SH3, SH4, and SH5) could be divided into two subgroups. Group 2A included phage SH3, which was close to coliphage K1F, while group 2B was comprised of phages SH4 and SH5, which are similar to *Cronobacter* phage Dev2.

The genomes of the five isolated phages are co-linear and share the same genomic organization as phage T7 with what

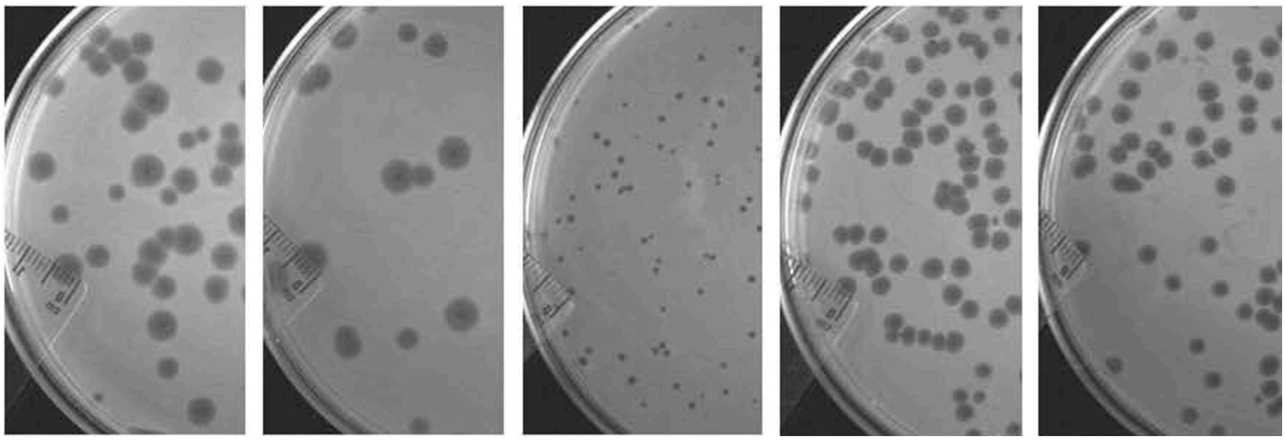

FIGURE 2 | Plaques formed by phages SH1, SH2, SH3, SH4, and SH5, respectively, from left to right on their host strains of *C. freundii* after an overnight incubation at 37°C.

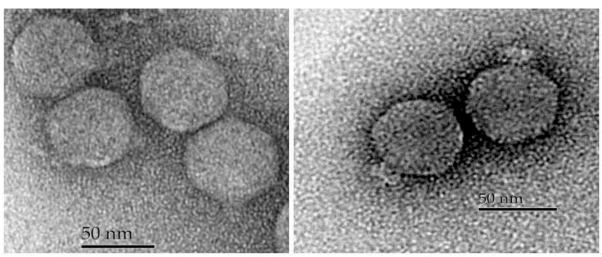

FIGURE 3 | Electron micrographs of phages SH1 (left) and SH3 (right).

seems to be early-, middle-, and late-expressed regions. The early genes are usually involved in host takeover and conversion of the host metabolism for the benefit of phage production (Pajunen et al., 2001). This region is also characterized by the presence of an RNA polymerase responsible for the transcription of all the middle- and late-expressed genes. The middle-expressed region includes genes responsible for DNA metabolism while the late region contains genes coding for structural proteins.

Proteomic Analyses

The structural proteome of one phage representing each of the three subgroups (phage SH1 for group 1, SH3 for group 2A and SH4 for group 2B) was analyzed. Purified phages were analyzed by LC-MS/MS and the results are presented in **Table 6**. For phage SH1, 11 proteins were detected with an amino acid coverage ranging from 12 to 65%. Ten of the 11 genes coding for these proteins were located in the presumably late-expressed module, as expected for genes coding for structural proteins. The other protein (ORF19) was a N-acetylmuramoyl-L-alanine amidase probably involved in host lysis and it had the lowest coverage (12%). Its gene was located in the middle-expressed region. It is unclear if this protein is in the phage structure or if it is a non-structural phage protein that was carried over from the phage purification process.

TABLE 3 | Morphological and genomic characteristics of the five isolated phages and phage T7.

Phage	Capsid (nm)	Tail (nm)		Genome size (bp)	GC%	Terminal repeat (bp)
		Width	Length			
SH1	61 ± 1.6	14 ± 0.9	12 ± 1.0	39,434	51.0	230
SH2	58 ± 2.5	14 ± 1.4	10 ± 1.1	39,158	50.7	242
SH3	65 ± 1.4	21 ± 1.1	13 ± 1.0	39,444	50.6	183
SH4	67 ± 1.3	29 ± 2.4	16 ± 1.2	39,274	52.6	190
SH5	65 ± 1.1	27 ± 1.8	16 ± 2.0	39,832	52.5	190
T7*	56	14	9	39,936	50	160

**According to (Ackermann and Nguyen, 1983; Dunn et al., 1983).*

For phage SH3, 9 structural proteins were detected with coverage ranging from 21 to 67%, while for phage SH4, 7 structural proteins were identified with coverage ranging from 18 to 40%. For these two phages, all the proteins detected were structural proteins from the capsid, head-tail joining, tail, tail tube, and tail fibers.

DNA Polymerase Phylogeny

Because the five *Citrobacter* podophages belong to the *T7virus* genus, we compared in greater detail their relationships with other characterized similar phages available in public database (**Figure 6**). The T7 DNA polymerase is a conserved protein often used to study the global distribution and diversity of podophages, in a manner analogous to the 16S rRNA in bacteria (Breitbart et al., 2004). Based on DNA polymerase phylogeny, the five phages were confirmed to belong to the *T7virus* genus in the subfamily *Autographivirinae*. However, they mapped at two different sub-branches. Phages SH1 and SH2 were similar to *Yersinia* phages phiYeO3-12 and vBYenP AP5, *Salmonella* phage phiSG-JL2, *Citrobacter* phage phiCFP-1, and *Enterobacter* phages E3 and E4. They were also closer to the prototype phage T7 than the other three phages characterized here. Phages SH3, SH4, and

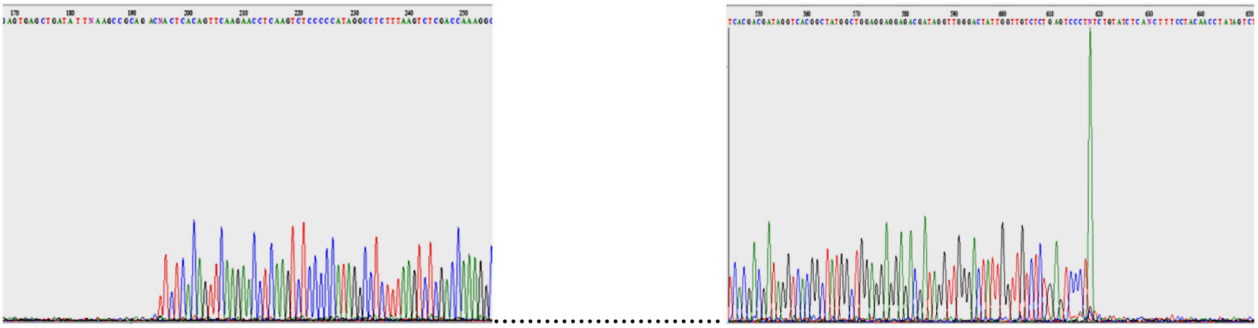

FIGURE 4 | SH5 genome sequencing with both reverse and forward primers.

SH5 were part of the same clade of t7viruses as SH1 and SH2, but clustered in different subgroups. Phage SH3 was related to *Enterobacteria* phages K1F and EcoDS1, and *Escherichia* phage PE3-1. Phages SH4 and SH5 were more related to *Cronobacter* phage Dev2. Taken altogether, despite the differences between these two groupings, SH1/SH2 and SH3/SH4/SH5 seem to be derived from a common ancestor.

Comparison Between Phages SH1/SH2, phiYeO3-12, and Coliphage T7 (Group 1)

Pairwise analyses between the deduced proteomes of phages SH1 and SH2 revealed 31 proteins (out of 53) with more than 95% identity (**Table 4**). Among them, seven (ORF8, ORF18, ORF23, ORF26, ORF29, ORF39, ORF44) were 100% identical, including two proteins with predicted functions, ORF39/internal virion protein B and ORF44/DNA packaging protein A. Phage SH1 also shared more than 95% identity with 31 proteins of *Yersinia* phage phiYeO3-12 including seven proteins with 100% identity (ORF8, ORF20, ORF22, ORF23, ORF25, ORF26, ORF29). Phage SH2 shared more than 95% identity with 34 proteins of *Yersinia* phage phiYeO3-12 including 13 proteins with 100% identity (**Table 4**). Phage SH2 seems more related to phage phiYeO3-12 than phage SH1. One of the most notable differences between phages SH1 and SH2/phiYeO3-12 was mobile elements. Phage SH1 is missing the homing endonuclease encoded on phages SH2 and phiYeO3-12 (ORF23^{SH2}/ORF5.3 $^{phiYeO3-12}$). However, phage SH1 has another homing endonuclease (ORF17), which is absent in both genomes of SH2 and phiYeO3-12. ORF17^{SH1} is homologous to a homing endonuclease found on *C. rodentium* phage CR44b (46%). Phage SH1 is also missing ORF35^{SH2}/ORF10B$^{phiYeO3-12}$ a minor capsid protein (Condron et al., 1991). On the other hand, the tail fiber protein of phage phiYeO3-12 shares only 68 and 67% identity with the tail fiber proteins of SH2 and SH1, respectively, which could explain the divergent host ranges between SH1/SH2 and phiYeO3-12. In fact, phages SH1 and SH2 were not able to infect the host strain *Yersinia enterocolitica* 6471/76-c (HER1249) of phage phiYeO3-12.

In addition, phages SH1 and SH2 shared 11 proteins with more than 80% amino acid identity with coliphage T7, including the RNA polymerase (ORF1^{T7} and ORF6$^{SH1/SH2}$). The T7 RNA polymerase initiates transcription by exclusively recognizing its own promoters to ensure fast and efficient transcription of phage DNA. It is also involved in DNA replication, maturation and packaging (Studier and Moffatt, 1986; Zhang and Studier, 2004).

Another T7 protein homologous to SH1/SH2 proteins was ORF2.5^{T7} (homologous to ORF16^{SH1} and ORF15^{SH2}), which is a single-stranded DNA binding protein. The *orf2.5*^{T7} gene is essential for phage DNA replication and recombination (Scaltriti et al., 2009, 2013). The N-acetylmuramoyl-L-alanine amidase ORF3.5^{T7} was also related to ORF19^{SH1} and ORF17^{SH2}. This lysozyme is involved in cell lysis but may also inhibit transcription by binding to the RNA polymerase to ensure a controlled burst of late transcription (Inouye et al., 1973; Moffatt and Studier, 1987). ORF21^{SH1} and ORF19^{SH2} were similar to the T7 primase/helicase, ORF4^{T7}. This primase/helicase activity is essential for DNA replication (Rosenberg et al., 1992) as the helicase catalyzes strand displacement during DNA replication while the primase is involved in the synthesis of the DNA lagging-strand (Mendelman et al., 1992).

The ORF5.7 protein of phage T7 shared a high level of identity with ORF26^{SH1} and ORF25^{SH2}. ORF5.7 stimulates the expression of gene 5.5 which encodes a H-NS binding protein (Zhu et al., 2012). When gene 5.5 is missing, the phage plaque and the burst sizes are reduced (Owen-Hughes et al., 1992; Liu and Richardson, 1993). The H-NS binding protein inhibits the function of the highly conserved host histone-like nucleoid structuring (H-NS) protein, which influences gene expression, recombination and transcription.

A notable difference between phage T7 and phages SH1/SH2 was in their antirestriction proteins (gp0.3^{T7}/ORF1$^{SH1/SH2}$). Restriction-modification (R-M) systems are well-known resistance mechanisms used by bacteria to block phage replication (Labrie et al., 2010). Phages also have several means to bypass these systems (Samson et al., 2013). The Phage T7Ocr (overcoming classical restriction, ORF0.3) protein mimics the DNA phosphate backbone, interacting directly with the type R-M*Eco*KI enzyme, and interfering with the activity of this system (Atanasiu et al., 2002; Stephanou et al., 2009). At the same genomic location (**Figure 5**), the phage SH1 and SH2 *orf1* genes code for a putative S-adenosyl-l-methionine hydrolase, homologous to gp0.3$^{phiYeO3-12}$, which destroys *S*-adenosyl-l-methionine, an essential R–M cofactor (Studier and Movva, 1976). The Ocr protein of

TABLE 4 | Features of the ORFs of phage SH2, identity with SH1, predicted functions of proteins, and best matches with database.

ORF	SH1 ORF,%	Predicted protein					SD sequence TAAGGAGGT (5′-3′)[a]	Predicted function	BLAST (extent; %aa identity)[b]	Aligned protein		
		Start (bp)	Stop (bp)	Size (aa)	MW(kDa)	pI				*E* Value	Size (aa)	Accession number
1	ORF1, 99	948	1406	152	17.0	7.6	TGAGGTAACcaccaa**ATG**	S-adenosyl-l-methionine hydrolase	gp0.3 [*Yersinia* phage phiYeO3-12] (152/152; 100%)	4.00E-110	152	NP_052065.1
2	ORF2, 87	1479	1679	66	7.5	6.8	ATAGGACTAacacc**ATG**		gp0.45 [*Salmonella* phage phiSG-JL2] (65/66; 98%)	4.00E-41	66	YP_001949746.1
3	ORF3, 74	1699	1857	52	6.0	9.7	ACAGGAGGAttagca**ATG**		hypothetical protein [*Enterobacter* phage E-4] (49/52; 94%)	3.00E-26	52	AKA61646.1
4	ORF4, 94	1854	2051	65	7.8	10.5	TGGTGAAACacgc**ATG**		hypothetical protein [*Enterobacter* phage E-4] (63/65; 97%)	2.00E-38	65	AKA61645.1
5	ORF5, 95	2073	3182	369	42.3	7.1	TAAGGACACactgaa**ATG**	Protein kinase	gp0.7 [*Yersinia* phage phiYeO3-12] (346/369; 94%)	0.0	369	NP_052070.1
6	ORF6, 99	3253	5907	884	98.8	7.1	CAATGAGGTaagca**ATG**	RNA polymerase	DNA-directed RNA polymerase [*Enterobacter* phage E-2] (881/884; 99%)	0.0	884	AKA61565.1
7	ORF7, 90	6008	6502	164	19.6	9.2	TAAGAGGATtactttA**TG**		gp1.05 [*Salmonella* phage phiSG-JL2] (76/165; 46%)	3.00E-38	169	YP_001949751.1
8	ORF8, 100	6593	6733	46	5.9	10.9	TAAGATACT**ATG**		gp1.1 [*Yersinia* phage phiYeO3-12] (46/46; 100%)	2.00E-23	46	NP_052073.1
9	ORF9, 69	6736	7008	91	10.3	7.9	AGTGGAACTaatg**ATG**	Deoxyguanosine triphospho-hydrolase inhibitor	gp1.2 [*Salmonella* phage phiSG-JL2] (90/91; 99%)	1.00E-160	92	YP_001949753.1
10	ORF10, 98	7103	8119	338	38.4	5.0	TGAGGAACAaccgt**ATG**	DNA ligase	gp1.3 [*Salmonella* phage phiSG-JL2] (333/338; 99%)	0.0	338	YP_001949754.1
11	ORF11, 94	8291	8548	85	9.9	11.2	TAAGGAGACaacatc**ATG**	LysR family transcriptional regulator	gp1.6 [*Yersinia* phage phiYeO3-12] (85/85; 100%)	2.00E-53	85	NP_052078.1
12	ORF12, 66	8548	9132	194	21.6	9.1	TAAGGAGGTgctgta**ATG**	Nucleotide kinase	phiYe-F10_00014 [*Yersinia* phage phiYe-F10] (163/190; 86%)	6.00E-115	190	AKQ06773.1
13	ORF14, 85	9119	9256	45	5.3	5.2	TAAGGGGCTgtgct**ATG**		AVU28_gp19 [*Enterobacter* phage E-3] (42/45; 93%)	1.00E-21	45	AKA61598.1
14	ORF15, 62	9253	9489	78	8.8	4.8	TAAGGAGGCcaataa**ATG**	Bacterial RNA polymerase inhibitor	ORF13 [*Yersinia* phage vB_YenP_AP5] (77/78; 99%)	1.00E-50	78	AIM40358.1
15	ORF16, 98	9542	10240	232	26.0	4.8	AAAGGAGAAacatc**ATG**	Single-stranded DNA-binding	phiYe-F10_00017 [*Yersinia* phage phiYe-F10] (227/232; 98%)	4.00E-166	232	AKQ06776.1
16	ORF18, 73	10240	10701	153	17.6	9.5	CGAGGACTTcta**ATG**	Endonuclease	gp3 [*Yersinia* phage phiYeO3-12] (152/153; 99%)	2.00E-105	153	NP_052083.1

(Continued)

TABLE 4 | Continued

ORF	SH1 ORF,%	Predicted protein								Aligned protein		
		Start (bp)	Stop (bp)	Size (aa)	MW(kDa)	pI	SD sequence TAAGGAGGT (5′-3′)[a]	Predicted function	BLAST (extent; %aa identity)[b]	*E* Value	Size (aa)	Accession number
17	ORF19, 99	10694	11149	151	16.9	9.0	TAAAGAAAA**ATG**	N-acetylmuramoyl-l-alanine amidase	AVU28_gp15 [*Enterobacter* phage E-3] (151/151; 100%)	2.00E-108	151	AKA61594.1
18	ORF20, 100	11154	11261	35	4.2	8.5	GAGGGTGATacc**ATG**		3.7 protein [*Yersinia* phage phiYeO3-12] (35/35; 100%)	2.00E-15	35	NP_052086.1
19	ORF21,99	11328	13028	566	6.3	5.2	TAAGGAATGtaca**ATG**	Primase/Helicase	gp4A [*Salmonella* phage phiSG-JL2] (560/566; 99%)	0.0	566	YP_001949764.1
19.1	ORF21.1, 94	11362	11517	51	5.6	6.5	TCTTTCTGTttc**ATG**		hypothetical protein [*Enterobacteria* phage T3] (49/51; 96%)	1.00E-26	51	AGM10719.1
19B	ORF21B,99	11514	13028	504	55.9	5.1	GGAGGCAGTaaccct**ATG**	Primase/Helicase	Primase/Helicase protein [*Enterobacter* phage E-3] (501/504; 99%)	0.0	504	AKA61593.1
19.2	ORF21.2, 97	12748	13077	109	12.1	6.7	GAAGGGAAAaccac**ATG**		gp4.2 [*Enterobacteria* phage T3] (103/109; 94%)	2.00E-66	109	NP_523317.1
20	ORF22, 97	13124	13336	70	7.7	10.0	ATAGGAGACacatc**ATG**		gp4.3 [*Yersinia* phage phiYeO3-12] (68/70; 97%)	4.00E-29	70	NP_052091.1
21	ORF23, 100	13349	13633	94	10.7	9.9	TAAGGAGCGaacact**ATG**		gp4.5 [*Yersinia* phage phiYeO3-12] (94/94; 100%)	2.00E-62	94	NP_052092.1
22	ORF24, 98	13701	15815	704	79.8	6.5	AAAGGAGGGcatt**ATG**	DNA polymerase	gp5 [*Yersinia* phage phiYeO3-12] (700/704; 99%)	0.0	704	NP_052093.1
23		15825	16157	110	13.0	9.5	TAAGGAGGAttt**ATG**	Homing Endonuclease	gp5.3 [*Yersinia* phage phiYeO3-12] (110/110; 100%)	2.00E-75	110	NP_052095.1
24	ORF25, 99	16135	16437	101	11.1	6.3	AAAGGAGAAacatt**ATG**	HNS binding	gp5.5 [*Yersinia* phage phiYeO3-12] (100/101; 99%)	4.00E-66	101	NP_052097.1
25	ORF26, 100	16553	16762	69	7.3	9.8	TTGGGAGGTactcta**ATG**		gp5.7 [*Yersinia* phage phiYeO3-12] (69/69; 100%)	8.00E-42	69	NP_052098.1
26	ORF27, 99	16705	16941	60	8.8	4.2	CAATGGTGGagc**ATG**		gp5.9 [*Yersinia* phage phiYeO3-12] (60/60; 100%)	1.00E-34	60	NP_072071.1
27	ORF28, 99	16938	17849	303	34.7	4.9	GGAGGATGAcga**ATG**	Exonuclease	AVU28_gp07 [*Enterobacter* phage E-3] (301/303; 99%)	0.0	303	AKA61586.1
28	ORF29, 100	17831	17944	37	4.1	9.7	CAAGGAGATttactt**ATG**		gp6.3 [*Yersinia* phage phiYeO3-12] (37/37; 100%)	1.00E-15	37	NP_052102.1
29	ORF30, 96	18039	18284	81	9.3	5.9	TTAAGAGGTgaattt**ATG**		gp6.5 [*Yersinia* phage phiYeO3-12] (79/81; 98%)	4.00E-51	81	NP_052103.1
30	ORF31, 69	18289	18540	83	8.8	9.1	ACAGGAGTAattat**ATG**	Head	gp6.7 [*Yersinia* phage vB_YenP_AP5] (83/83; 100%)	2.00E-49	83	YP_009102822.1
31	ORF32, 96	18568	18888	106	11.0	9.8	TAGGGAGAAacatc**ATG**	Host specificity protein B	gp7.3 [*Salmonella* phage phiSG-JL2] (105/106; 99%)	1.00E-62	106	YP_001949779.1

(Continued)

TABLE 4 | Continued

ORF	SH1 ORF,%	Start (bp)	Stop (bp)	Predicted protein			SD sequence TAAGGAGGT (5′-3′)[a]	Predicted function	BLAST (extent; %aa identity)[b]	Aligned protein		
				Size (aa)	MW(kDa)	pI				*E* Value	Size (aa)	Accession number
32	ORF33, 99	18899	20506	535	58.6	4.5	TAAGGAGGActga**ATG**	Head-to-tail joining	gp8 [*Yersinia* phage phiYeO3-12] (535/535; 100%)	0.0	535	NP_052106.1
33	ORF34, 97	20608	21540	310	33.8	4.3	TTAGGAGATttaaca**ATG**	Capsid assembly	ORF30 [*Citrobacter* phage phiCFP-1] (303/310; 98%)	0.0	310	AKA62148.1
34	ORF35, 95	21697	22740	347	36.8	6.2	TAAGGAGATtcaac**ATG**	Minor and Major capsid	10A [*Yersinia* phage phiYe-F10] (344/346; 99%)	0.0	347	AKQ06793.1
35		22779	23012	77	7.4	4.5	TCAGAAGACt**ATG**	Minor capsid	AVU28_gp35 [*Enterobacter* phage E-3] (73/77; 95%)	1.00E-39	77	AKA61614.1
36	ORF36, 99	23125	23715	196	22.2	4.5	ACAGGAGGTaacatc**ATG**	Tail tubular A	gp11 [*Yersinia* phage phiYeO3-12] (196/196; 100%)	1.00E-141	196	NP_052110.1
37	ORF37, 99	23731	26136	801	89.8	5.9	CAAGGAGGCtct**ATG**	Tail tubular B	gp12 [*Salmonella* phage phiSG-JL2] (797/801; 99%)	0.0	801	YP_001949785.1
38	ORF38, 98	26209	26619	136	15.8	5.6	TAAAGCATT**ATG**	Internal virion A	AXI78_gp37 [*Enterobacter* phage E-2] (134/136; 99%)	4.00E-95	136	AKA61575.1
39	ORF39, 100	26622	27215	197	21.2	9.4	GTAGGAGGTaact**ATG**	Internal virion B	gp14 [*Yersinia* phage phiYeO3-12] (194/197; 98%)	4.00E-136	197	NP_052114.1
40	ORF40, 80	27218	29461	747	84.6	6.1	CCGGGAGGTaata**ATG**	Internal virion C	ORF37 [*Citrobacter* phage phiCFP-1] (711/747; 95%)	0.0	747	AKA62155.1
41	ORF41, 83	29484	33452	1322	144.2	6.7	TAAGGAGGCtcc**ATG**	Internal virion D	ORF38 [*Citrobacter* phage phiCFP-1] (1296/1322; 98%)	0.0	1322	AKA62156.1
42	ORF42, 92	33524	35500	658	69.9	6.0	AAAGGAGGTcac**ATG**	Tail fiber	gp17 [*Salmonella* phage phiSG-JL2] (593/658; 90%)	0.0	658	YP_001949790.1
43	ORF43, 99	35511	35714	67	7.4	6.1	TAAGGAGGAcata**ATG**	Lysis	gp17.5 [*Yersinia* phage phiYeO3-12] (66/67; 99%)	5.00E-39	67	NP_052118.1
44	ORF44, 100	35718	35984	88	9.9	4.7	CAAGGAGTAacct**ATG**	DNA packaging A	gp18 [*Salmonella* phage phiSG-JL2] (88/88; 100%)	1.00E-55	88	YP_001949792.1
45	ORF45, 99	36062	36526	150	17.3	9.2	ATGGGAGGTgtt**ATG**	Endopeptidase Rz	ORF42 [*Citrobacter* phage phiCFP-1] (152/154; 99%)	1.00E-107	154	AKA62160.1
45.7	ORF45.7, 99	36189	36443	84	9.3	9.8	TAATCCAAA**ATG**		gp18.7 [*Salmonella* phage phiSG-JL2] (83/84; 99%)	9.00E-52	84	YP_001949794.1
46	ORF46, 99	36501	38264	587	66.6	5.3	TAAGGAGATgcaga**ATG**	DNA packaging B	gp19 [*Salmonella* phage phiSG-JL2] (581/587; 99%)	0.0	587	YP_001949795.1
46.2	ORF46.2, 93	37213	37383	56	6.1	10.0	GAAGACTTGtact**ATG**		19.2 protein [*Yersinia* phage phiYeO3-12] (56/56; 100%)	2.00E-28	77	NP_052123.1
46.3	ORF46.3, 95	37687	37815	42	4.7	11.9	TGGCGGGTTccgcg**ATG**		19.3 protein [*Yersinia* phage phiYeO3-12] (42/42; 100%)	1.00E-19	42	NP_052124.1
47	ORF47, 96	38509	38658	49	5.5	7.9	AAAGGAGGTggctcA**ATG**		AVU28_gp23 [*Enterobacter* phage E-3] (48/49; 98%)	9.00E-25	49	AKA61602.1

[a] *Start codon indicated in boldface; Match to SD sequence is indicated by underlining; SD position is indicated in uppercase.*

[b] *The number of identical amino acids/The total number of amino acids of smallest protein.*

TABLE 5 | Features of the ORFs of phage SH5, identity with SH4 and SH3, predicted functions of proteins, and best matches with database.

ORF	SH4 ORF,%	SH3 ORF, %	Predicted protein							Aligned protein		
			Start	Stop	Size(aa)	MW(kDa)	pI	SD sequence (TAAGGAGGT) (5′-3′)[a]	Predicted function	Blast (extent; %aa identity)[b]	*E* Value	Size (aa)	Accession number
1	ORF1, 100		934	1137	68	7.9	6.1	ATAGGATAAacaag**ATG**		metaG-MbCM1_078 [*Synechococcus* phage metaG-MbCM1] (28/59; 47%)	3.00E-11	59	YP_007001569.1
2	ORF2, 100		1134	1658	175	20.2	5.7	TAAGGAACTacaatc**ATG**		CPT_Seurat66 [*Escherichia* phage Seurat](87/167; 52%)	6.00E-48	167	YP_009152010.1
3	ORF3, 100	ORF3, 84	1652	1807	52	5.9	9.4	AGGTGAGGTcatcaag**ATG**		gp0.35 [*Enterobacteria* phage EcoDS1] (47/50; 94%)	2.00E-27	50	YP_002003737.1
4	ORF4, 100		2006	2173	56	5.9	8.3	ATAGGAGTTaact**ATG**		PE3_004 [*Escherichia* phage PE3-1] (48/55; 87%)	2.00E-24	55	YP_009044252.1
5	ORF5, 100	ORF5, 99	2177	2374	66	7.5	11.0	GCGGGATAAacc**ATG**		gp0.6 [*Enterobacteria* phage EcoDS1](64/65; 98%)	4.00E-37	65	YP_002003739.1
6	ORF6, 100	ORF6, 56	2374	2700	108	12.1	9.3	TTGGGAGCAaactgta**ATG**		PE3_006 [*Escherichia* phage PE3-1] (76/108; 70%)	8.00E-48	130	YP_009044254.1
7	ORF7, 100	ORF7, 93	2798	5479	894	100.5	7.6	CAAGGACTTtaagt**ATG**	RNA polymerase	gp1 [*Cronobacter* phage Dev2] (883/893; 99%)	0.0	893	YP_009005115.1
8	ORF8, 100	ORF8, 78	5492	5692	67	7.3	9.7	TAAGGAGGCatctac**ATG**		gp1.1 [*Cronobacter* phage Dev2] (66/66; 100%)	6.00E-38	66	YP_009005116.1
9	ORF9, 100		5771	6250	160	18.6	9.3	AGAGGTTGAcact**ATG**		gp1.06 [*Cronobacter* phage Dev2](155/159; 97%)	1.00E-110	159	YP_009005117.1
10	ORF10, 100	ORF10, 98	6339	6518	60	6.8	10.2	ACTGGAGATttaacc**ATG**		gp1.15 [*Cronobacter* phage Dev2](58/59; 98%)	4.00E-33	59	YP_009005118.1
11	ORF11, 100	ORF11, 22	6522	6809	96	11.2	6.3	GTAGGAGCGtaagac**ATG**		PE3_010 [*Escherichia* phage PE3-1](81/95; 85%)	5.00E-56	95	YP_009044258.1
12	ORF12, 99	ORF12, 75	6827	7897	357	40.2	5.5	TCTGGAGACattaacg**ATG**	DNA ligase	gp1.3 [*Enterobacteria* phage EcoDS1] (318/357; 89%)	0.0	365	YP_002003747.1
13	ORF13, 100	ORF13, 49	8019	8273	85	9.85	9.9	AGAGGAGAAaccttA**TG**		gp1.6 [*Enterobacteria* phage EcoDS1] (73/84; 87%)	6.00E-47	84	YP_002003748.1
14	ORF14, 100	ORF14, 67	8273	8593	107	12.2	6.9	CAAGGAGGAgttcta**ATG**		gp1.7 [*Cronobacter* phage Dev2] (85/107; 79%)	5.00E-50	116	YP_009005122.1
15	ORF15, 100	ORF16, 65	8672	8887	72	8.2	4.5	GAAGGAGAAaggact**ATG**	Bacterial RNA polymerase inhibitor	gp2 [*Cronobacter* phage Dev2] (48/54; 89%)	3.00E-25	54	YP_009005123.1

(Continued)

TABLE 5 | Continued

ORF	SH4 ORF,%	SH3 ORF, %	Predicted protein							Aligned protein		
			Start	Stop	Size(aa)	MW(kDa)	pI	SD sequence (TAAGGAGGT) (5′-3′)[a]	Predicted function	Blast (extent; %aa identity)[b]	*E* Value	Size (aa)	Accession number
16	ORF16, 100	ORF17, 85	8935	9633	233	25.4	4.8	CTAGGAGATttacaccg**ATG**	Helix-destabilizing protein	gp2.5 [*Cronobacter* phage Dev2] (229/232; 99%)	2.00E-164	232	YP_009005124.1
17	ORF17, 100	ORF18, 43	9870	10088	72	8.3	9.9	TAAGAAGCAt**ATG**	Endonuclease	gp3 [*Cronobacter* phage Dev2] (72/72; 100%)	2.00E-44	139	YP_009005125.1
18	ORF18, 100	ORF19, 81	10085	10309	75	8.6	9.7	AAAGGAGCTaagaa**ATG**		gp3.2 [*Cronobacter* phage Dev2] (74/74; 100%)	3.00E-45	74	YP_009005126.1
19	ORF19, 98	ORF20, 90	10299	10757	153	16.9	8.8	GCTGGTGGTgtaca**ATG**	N-acetylmuramoyl-l-alanine amidase	gp3.5 [*Cronobacter* phage Dev2] (152/152; 100%)	8.00E-109	152	YP_009005127.1
20	ORF20, 100	ORF21, 61	10772	10984	71	7.4	10.1	CAAGGAGTAttaac**ATG**		gp3.7 [*Cronobacter* phage Dev2] (69/70; 99%)	1.00E-28	70	YP_009005128.1
21			11142	11507	122	13.9	10.1	GCGGGATAAacc**ATG**	HNH endonuclease	gp3.8 [*Enterobacteria* phage T7] (65/121; 54%)	1.00E-40	121	NP_041974.1
22	ORF21, 97	ORF22, 90	11482	13179	566	62.2	5.1	TAAGGAGGCtc**ATG**	Primase/ Helicase	gp4 [*Cronobacter* phage Dev2] (545/566; 96%)	0.0	567	YP_009005129.1
22B	ORF21B, 98	ORF22B, 92	11782	13179	465	51.3	5.2	TTGGGTAGGc**ATG**	Primase/ Helicase	gp4 [Cronobacter phage Dev2] (475/465; 98%)	0.0	567	YP_009005129.1
22.2	ORF21.2, 61	ORF22.2, 62	12908	13255	115	13.0	9.0	AAAGGTAAGtctc**ATG**		gp4.2 [Enterobacteria phage K1F] (63/107; 56%)	2.00E-25	107	CAJ29367.1
23	ORF22, 66	ORF23, 69	13182	13766	194	21.2	4.7	CAACGACTTctgacc**ATG**		gp4.1 [*Cronobacter* phage Dev2] (168/177; 95%)	1.00E-117	177	YP_009005130.1
24	ORF23, 99	ORF24, 90	13837	16008	724	80.9	7.0	ATAGGAGACatt**ATG**	DNA polymerase	gp5 [*Cronobacter* phage Dev2] (717/723; 99%)	0.0	723	YP_009005131.1
25	ORF24, 97	ORF26, 96	16008	16292	95	10.5	5.2	GAAGGAGTGtcacta**ATG**	HNS binding protein	gp5.5 [*Cronobacter* phage Dev2] (92/94; 98%)	4.00E-58	94	YP_009005133.1
26	ORF25, 100	ORF27, 100	16289	16498	70	7.4	9.0	ATTCGAGGTcaaacg**ATG**		gp21 [*Enterobacteria* phage K1F] (69/69; 100%)	5.00E-43	69	YP_338112.1
27	ORF26, 100		16495	16770	92	9.9	5.3	GGAGGCTGTct**ATG**		ASC_0027 [*Klebsiella* phage K11](37/68; 54%)	2.00E-19	68	YP_002003815.1
28	ORF27, 99	ORF28, 90	16763	17629	289	32.9	5.4	AAAGGAGGTctgcggg**ATG**	Exonuclease	gp6 [*Cronobacter* phage Dev2] (284/288; 99%)	0.0	288	YP_009005135.1
29	ORF28, 100	ORF29, 56	17837	18109	91	9.9	5.2	AGAGGAGACtttaag**ATG**		gp6.5 [*Cronobacter* phage Dev2] (90/90; 100%)	8.00E-58	90	YP_009005136.1
30	ORF29, 100	ORF30, 92	18120	18344	75	7.6	6.2	AAAGGAGGGact**ATG**	Head protein	gp6.7 [*Cronobacter* phage Dev2] (74/74; 100%)	1.00E-42	74	YP_009005137.1

(Continued)

TABLE 5 | Continued

ORF	SH4 ORF,%	SH3 ORF, %	Predicted protein							Aligned protein		
			Start	Stop	Size(aa)	MW(kDa)	pI	SD sequence (TAAGGAGGT) (5′-3′)[a]	Predicted function	Blast (extent; %aa identity)[b]	*E* Value	Size (aa)	Accession number
31	ORF30, 100	ORF31, 91	18348	18752	135	15.5	6.1	ACATGGGGTAAGac**ATG**		gp34 [*Citrobacter* phage CR44b] (127/135; 94%)	7.00E-89	185	YP_009007168.1
32	ORF31, 100	ORF32, 96	19011	20579	523	57.2	4.6	GCAGGAGGTgacaa**ATG**	Head to tail connector protein	gp8 [*Cronobacter* phage Dev2] (522/522; 100%)	0.0	522	YP_009005140.1
33	ORF32, 99	ORF33, 81	20684	21565	294	31.7	4.4	AAAGGAGAAcgactca**ATG**	Capsid assembly protein	gp9 [*Cronobacter* phage Dev2] (290/293; 99%)	0.0	293	YP_009005141.1
34	ORF33, 99	ORF34, 94	21697	22746	350	36.4	5.8	ATAGGAGAAttatcat**ATG**	Major capsid protein	gp10 [*Cronobacter* phage Dev2] (347/349; 99%)	0.0	349	YP_009005142.1
35	ORF34, 100	ORF35, 97	23067	23633	189	21.3	4.4	TAAGGAGGGcct**ATG**	Tail tube protein A	gp11 [*Cronobacter* phage Dev2] (187/188; 99%)	3.00E-135	188	YP_009005144.1
36	ORF35, 99	ORF36, 87	23645	26014	790	87.6	5.8	ATAGGAGGTgat**ATG**	Tail tube protein B	gp12 [*Cronobacter* phage Dev2] (779/789; 99%)	0.0	789	YP_009005145.1
37	ORF36, 97	ORF37, 83	26090	26548	153	17.5	6.9	ATAGGAGACtttt**ATG**	Internal virion protein A	gp13 [*Cronobacter* phage Dev2] (150/152; 99%)	6.00E-107	152	YP_009005146.1
38	ORF37, 98	ORF38, 91	26669	27256	196	20.4	6.8	CCGGGAGGTgaaag**ATG**	Internal virion protein B	gp14 [*Cronobacter* phage Dev2] (194/195; 99%)	1.00E-136	195	YP_009005147.1
39	ORF38, 99	ORF39, 89	27268	29550	761	85.3	5.5	ATAGGAGGAcca**ATG**	Internal virion protein C	gp15 [*Cronobacter* phage Dev2] (749/760; 99%)	0.0	760	YP_009005148.1
40	ORF39, 99	ORF40, 92	29556	33452	1299	141.0	5.8	TAAGGAGTAataaca**ATG**	Internal virion protein D	gp16 [*Cronobacter* phage Dev2] (1282/1298; 99%)	0.0	1298	YP_009005149.1
41	ORF40, 100	ORF41, 79	33520	36018	832	91.3	6.4	TAAGGAGGCcca**ATG**	Tail fibers	gp17 [*Cronobacter* phage Dev2] (805/832; 97%)	0.0	832	YP_009005150.1
42	ORF41, 100	ORF42, 95	36065	36259	65	6.9	8.0	AACGGAGGTatt**ATG**	Lysis protein	gp17.5 [*Cronobacter* phage Dev2] (64/64; 100%)	4.00E-37	64	YP_009005151.1
43	ORF42, 100	ORF43, 97	36256	36519	87	10.1	4.8	AGTGGAGGTaagac**ATG**	DNA packaging protein	gp18 [*Cronobacter* phage Dev2] (87/87; 100%)	1.00E-54	87	YP_009005152.1
44	ORF43, 100	ORF44, 73	36624	37073	150	16.9	8.8	CGAGGAGGGcaact**ATG**	Endopeptidase Rz	gp18.5 [*Cronobacter* phage Dev2] (147/149; 99%)	3.00E-101	149	YP_009005153.1
44.7	ORF43.7, 100	ORF44.7, 72	36727	36999	90	9.8	9.6	GAAGGTAAGca**ATG**	Endopeptidase Rz1	gp18.7 [Enterobacteria phage EcoDS1] (60/89; 67%)	2.00E-31	91	YP_002003785.1
45	ORF44, 100	ORF45, 94	37103	38833	577	65.1	5.3	TCAGGCGCTt**ATG**	Maturation protein	gp19 [*Cronobacter* phage Dev2] (574/577; 99%)	0.0	587	YP_009005154.1
45.2	ORF44.2, 100		37752	37877	41	4.8	12.1	TATCCTCGTg**ATG**		gp19.2 [*Enterobacteria* phage K1F] (27/55; 49%)	7.00E-09	55	CAJ29396.1
46	ORF45, 100	ORF46, 94	39124	39282	53	5.5	9.3	GTATGTAGC**ATG**		gp19.5 [*Cronobacter* phage Dev2] (52/52; 100%)	2.00E-27	52	YP_009005155.1

[a] *Start codon indicated in boldface; Match to SD sequence is indicated by underlining; SD position is indicated in uppercase.*

[b] *The number of identical amino acids/The total number of amino acids of smallest protein.*

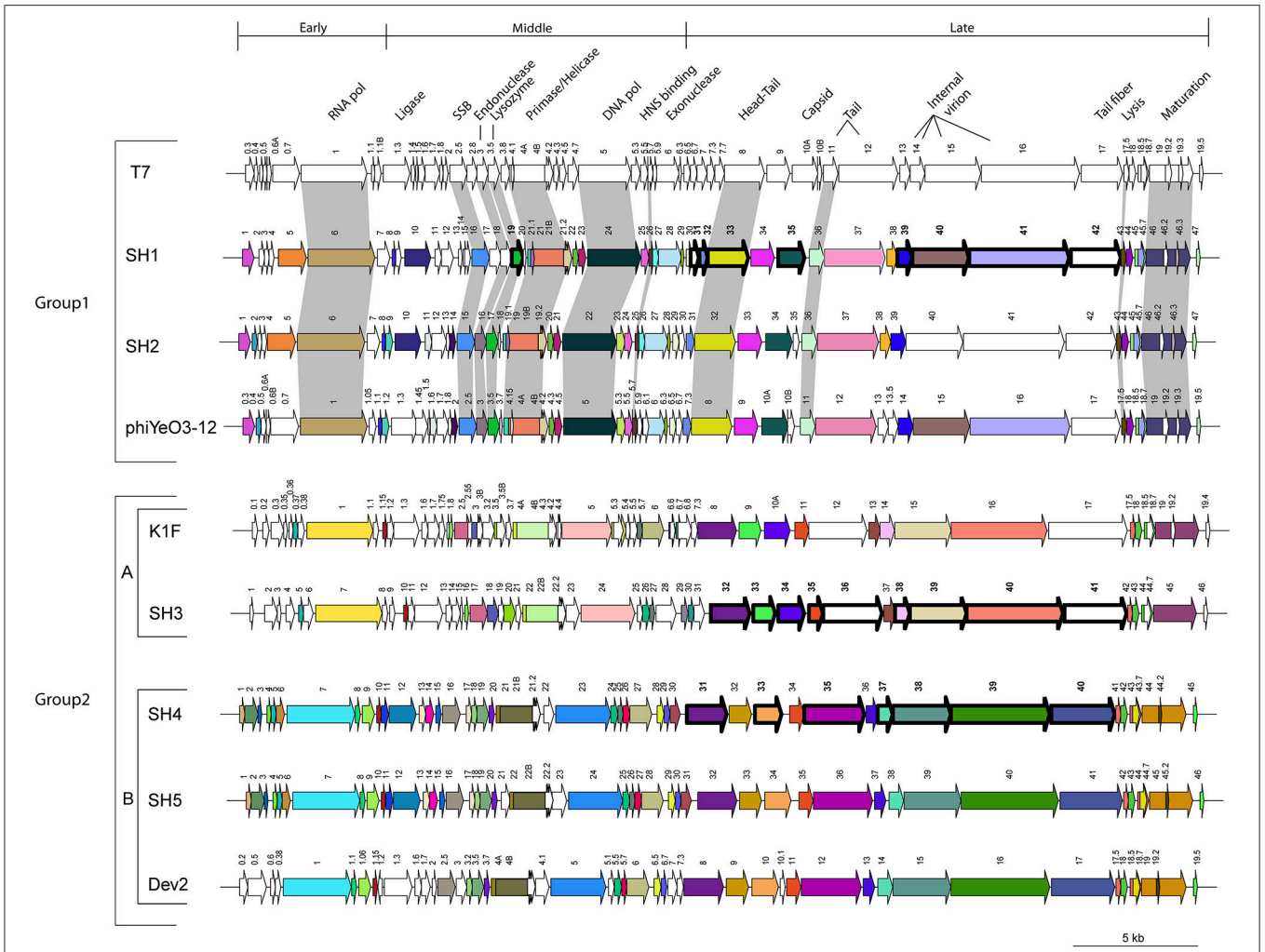

FIGURE 5 | Schematic representation of the genomic organization of phage T7 (NC_001604.1) compared to phages SH1, SH2, phiYeO3-12 (NC_001271.1), K1F (NC_007456.1), SH3, SH4, SH5, and Dev2 (NC_023558.1). Each arrow represents an ORF. Deduced ORFs sharing 95% amino acid identity are represented with the same color. Gray shading indicates ORFs whose translated products share 80% identity with the ones of phage T7. Finally, arrows with thick outlines and bold numbers represent structural proteins detected by LC-MS/MS.

phage T7 does not have the hydrolase activity. However, the Ocr protein of *E. coli* podophage T3, whose gene is located at the same genomic position, possesses this hydrolase activity.

Comparison between Phages SH3 and K1F (Group 2A)

The deduced proteome of phage SH3 (49 ORFs) ranged from 30 to 75% identity to the proteins of phages SH1 and SH2. However, phage SH3 had eight proteins with more than 95% identity to proteins of phages SH4 and SH5, including 100% identity between ORF27^{SH3} and ORF25^{SH4}/ORF26^{SH5} (**Table 5**). Otherwise, the closest phage to SH3 was coliphage K1F with 23 proteins sharing more than 95% identity. Of these, four proteins are 100% identical, including two with a known function (lysis protein and DNA packaging protein). Genetic differences were noted between *Citrobacter* phage SH3 and *E. coli* phage K1F and the most important difference lies in tail fibers (Gp17^{K1F}/ORF41^{SH3}) that consist of two domains. The N-terminal domain is responsible for attachment to the phage tail and the C-terminal domain is involved in the recognition of and adsorption to the host LPS (Kajsík et al., 2014). The N-terminal parts of the tail fibers of both K1F and SH3 shared a region with the phage T7 tail fiber. However, the central catalytic portion of Gp17^{K1F} encodes an endosialidase to penetrate the host polysaccharide capsule (Scholl and Merril, 2005) while ORF41^{SH3} contains a domain of the SGNH hydrolase superfamily like the tail fibers of phages Dev2, SH4, and SH5. However, the C-terminal part of ORF41^{SH3} is different than the tail fibers of phages SH4, SH5, and Dev2, which explains its different host range. The SH3 genome is also missing the putative group I intron present within the DNA polymerase of K1F (gp5.3) which encodes a homing endonuclease.

TABLE 6 | Identified peptides for phages SH1, SH3 and SH4 and their predicted functions.

Phage	Start	Stop	ORF	Predicted function	Mass (kDa)	Exclusive unique peptide	Coverage (%)
SH1	11614	12069	19	N-acetylmuramoyl-L-alanine amidase	17	2	12
	18713	19039	31	Capsid protein	12	2	36
	19067	19387	32	Host specficity protein B	11	3	29
	19398	21005	33	Capsid to tail joining protein	59	29	65
	22196	23236	35	Major capsid protein	37	22	60
	23426	24016	36	Tail tubular protein A	22	5	23
	24032	26437	37	Tail tubular protein B	90	32	44
	26923	27516	39	Internal virion protein B	21	12	65
	27519	29762	40	Internal virion protein C	85	36	59
	29781	33743	41	Internal virion protein D	144	63	57
	33815	35791	42	Tail fibers protein	70	21	48
SH3	18963	20531	32	Capsid to tail connector protein	57	26	67
	20676	21560	33	Capsid assembly protein	32	7	21
	21687	22730	34	Major capsid protein	36	8	50
	22925	23491	35	Tail tube protein A	21	5	32
	23503	25872	36	Tail tube protein B	88	24	37
	26404	26991	38	Internal virion protein B	20	10	65
	27003	29285	39	Internal virion protein C	85	25	42
	29290	33177	40	Internal virion protein D	141	45	45
	33243	35747	41	Tail fibers protein	91	20	33
SH4	18577	20145	31	Capsid to tail connector protein	57	5	39
	21263	22312	33	Major capsid protein	36	15	40
	23211	25580	35	Tail tube protein B	88	10	24
	26111	26698	37	Internal virion protein B	20	2	32
	26710	28992	38	Internal virion protein C	85	14	29
	28998	32894	39	Internal virion protein D	141	14	20
	32962	35460	40	Tail fibers protein	91	9	18

Comparison between Phages SH4/SH5 and Dev2 (Group 2B)

Of the 45 genes of phage SH5, 33 were 100% identical to genes of phage SH4. Ten of these genes are also 100% identical to the *T7virus Cronobacter* phage Dev2 genes. These conserved genes suggest that the three phages may be derived from a common ancestor. In addition, phages SH4 and SH5 have more than 95% aa identity with almost all of the phage Dev2 structural proteins. Interestingly, the putative tail fiber proteins ORF40^{SH4} and ORF41^{SH5} were 99% identical to tail fiber gp17 of phage Dev2, suggesting a similar host range. We received phage Dev2 and tested its host range in parallel with phages SH4 and SH5 on the 31 bacterial strains available. The three phages were able to lyse the same strains, *C. freundii* CF3, *C. freundii* CF4, and *C. turicensis* 290708/07.

Phages SH4 and SH5 are missing the genes coding for gp5.1- and gp10.1-like located in the late-expressed region, found in Dev2 (Kajsík et al., 2014). Most genomic differences between SH4/SH5 and Dev2 were located in the early-expressed region. ORF21 of phage SH5, which encodes an HNH endonuclease with a zinc-binding motif involved in different steps of phage development (Anba et al., 2002), was missing from phages SH4 and Dev2. However, ORF21 shares 54% identity with T7 gp3.8.

The SH4 and SH5 proteins with the lowest similarity were ORF22^{SH4} (132 aa) and ORF23^{SH5} (194 aa) but these were still 66% identical. Their amino acid sequences could be aligned perfectly at the C-terminal end but ORF22^{SH4} is missing the N-terminal portion of ORF23^{SH5}. A mutation may have occurred as we noticed the lack of a T base at the ATG codon of ORF22^{SH4}. ORF23^{SH5} had 95% identity to gp4.1 of phage Dev2 but its function is unknown.

DISCUSSION

In this study, we isolated and characterized five virulent *Podoviridae* phages infecting *C. freundii*, an emerging pathogenic bacterial species (Samonis et al., 2008). Genome analyses showed that the five newly isolated phages belong to the *Autographivirinae* subfamily and the *T7virus* genus. Their morphological and genomic properties allowed us to separate them into two different groups, group 1 (phages SH1 and SH2) and group 2 (phages SH3, SH4, and SH5). However, the two groups are co-linear and share conserved genomic

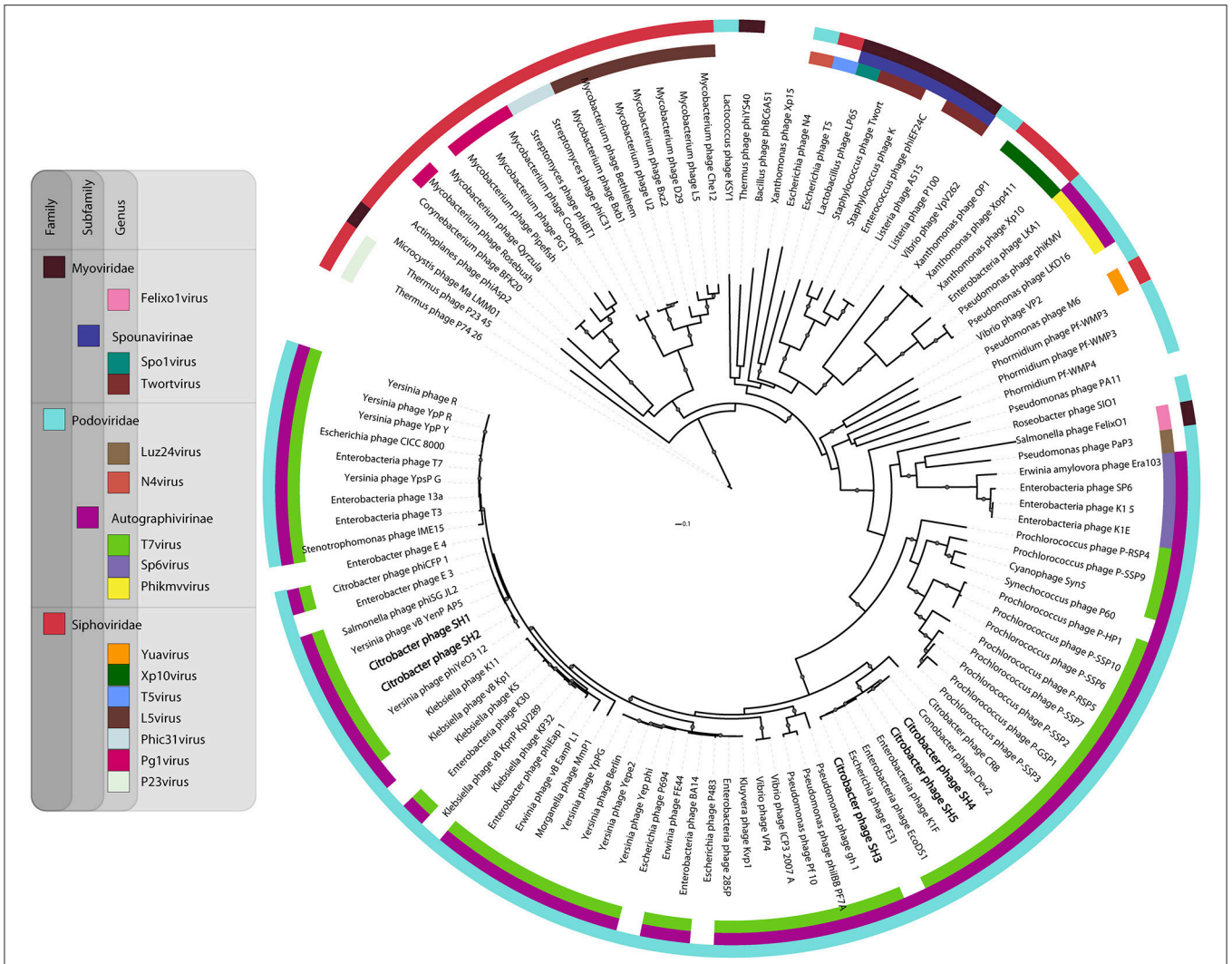

FIGURE 6 | Phylogenetic relationship between selected phage DNA polymerase sequences.

organization. They are flanked by terminal repeats involved in concatemer formation, DNA packaging, and particle maturation (Chung et al., 1990). Despite their small size (close to 40 kb), the five phage genomes encode the usual modules with genes coding for proteins involved in DNA replication, transcription regulation, morphological proteins, lysis proteins, as well as DNA maturation and packaging. As such, they have very compact genomes with overlapping genes (Mendelman et al., 1992) as more than 90% of the five genome sequences were predicted to encode proteins. For phages SH1, SH3, and SH4 almost all the predicted structural proteins were detected by LC-MS/MS, showing that they are indeed transcribed and translated.

Another reason for sequencing the new phage genomes is to provide a clearer view about the dynamics of phage populations over space and time. Based on genomic and proteomic identification, we could define evolutionary relationships between these podophages (Brüssow and Hendrix, 2002). For example, phage T7 was isolated in 1945 (Delbrück, 1945), phage phiYeO3-12 from sewage in 1988 in Finland (Al-Hendy et al., 1991), phage K1F from sewage in 1984 in the USA (Scholl and Merril, 2005), and phage Dev2 was recently isolated from sewage in Slovakia (Kajsík et al., 2014). All five *C. freundii* phages characterized in this study were isolated from different sewage samples collected in Tunisia in 2014. These phages are geographically and temporally distant but from an evolutionary perspective, these phages likely shared a common ancestor.

As phages tend to coevolve with their bacterial hosts (Skurnik and Strauch, 2006) and *C. freundii* can produce enterotoxins (Guarino et al., 1987), we inspected the five phage genomes for the presence of host related genes, particularly those coding for known virulence-factors or integrase. No such genes were found, indicating that they are truly lytic phages as well as suggesting that they may be safe for therapeutic or prevention applications. Moreover, it was relatively easy to purify them and we obtained highly concentrated phage preparations. Conversely, these phages were inactivated at very acidic pH (2–3), suggesting

that they may not survive in high numbers after passage through the gastrointestinal tract or in highly acidic foods. Others have shown that microencapsulation in alginate-chitosan microspheres significantly improved the survival and stability of phages under harsh acidic conditions (Ma et al., 2008). Finally, their limited host range suggests that they should be used in combination to maximize strain coverage. Of note, no CRISPR-Cas systems were found in the *C. freundii* genomes analyzed.

Taken altogether, the newly characterized *Podoviridae* phages SH1, SH2, SH3, SH4, and SH5 have appealing properties for prophylactic or therapeutic use to control the proliferation of *C. freundii* infections. The analyses of these *Citrobacter* phages also provided new evolutionary relationships with the expanding group of phages belonging to the *T7virus* genus, including with phages infecting *Cronobacter* and *Yersinia* species of the *Enterobacteriaceae* family.

AUTHOR CONTRIBUTIONS

SM, KS, RK conceived and designed the study and afforded the materials. SH performed the experiments, analyzed the data and drafted the manuscript. GR participated in the data analysis and helped in the coordination of the experiments. DT did the sequencing and the electron microscopy. SL designed the figures and helped in the bioinformatics analysis. SM critically revised the manuscript. All authors read and approved the manuscript.

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Genomics of Three New Bacteriophages Useful in the Biocontrol of *Salmonella*

*Carlota Bardina †, Joan Colom †, Denis A. Spricigo †, Jennifer Otero, Miquel Sánchez-Osuna, Pilar Cortés * and Montserrat Llagostera*

Departament de Genètica i de Microbiologia, Molecular Microbiology, Universitat Autònoma de Barcelona, Barcelona, Spain

Non-typhoid *Salmonella* is the principal pathogen related to food-borne diseases throughout the world. Widespread antibiotic resistance has adversely affected human health and has encouraged the search for alternative antimicrobial agents. The advances in bacteriophage therapy highlight their use in controlling a broad spectrum of food-borne pathogens. One requirement for the use of bacteriophages as antibacterials is the characterization of their genomes. In this work, complete genome sequencing and molecular analyses were carried out for three new virulent *Salmonella*-specific bacteriophages (UAB_Phi20, UAB_Phi78, and UAB_Phi87) able to infect a broad range of *Salmonella* strains. Sequence analysis of the genomes of UAB_Phi20, UAB_Phi78, and UAB_Phi87 bacteriophages did not evidence the presence of known virulence-associated and antibiotic resistance genes, and potential immunoreactive food allergens. The UAB_Phi20 genome comprised 41,809 base pairs with 80 open reading frames (ORFs); 24 of them with assigned function. Genome sequence showed a high homology of UAB_Phi20 with *Salmonella* bacteriophage P22 and other *P22likeviruses genus* of the *Podoviridae* family, including ST64T and ST104. The DNA of UAB_Phi78 contained 44,110 bp including direct terminal repeats (DTR) of 179 bp and 58 putative ORFs were predicted and 20 were assigned function. This bacteriophage was assigned to the *SP6likeviruses* genus of the *Podoviridae* family based on its high similarity not only with SP6 but also with the K1-5, K1E, and K1F bacteriophages, all of which infect *Escherichia coli.* The UAB_Phi87 genome sequence consisted of 87,669 bp with terminal direct repeats of 608 bp; although 148 ORFs were identified, putative functions could be assigned to only 29 of them. Sequence comparisons revealed the mosaic structure of UAB_Phi87 and its high similarity with bacteriophages Felix O1 and wV8 of *E. coli* with respect to genetic content and functional organization. Phylogenetic analysis of large terminase subunits confirms their packaging strategies and grouping to the different phage genus type. All these studies are necessary for the development and the use of an efficient cocktail with commercial applications in bacteriophage therapy against *Salmonella*.

Keywords: *Salmonella*, bacteriophage, genomics, chromosomal ends, *Myoviridae*, *Podoviridae*

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Sherwood Reid Casjens,
University of Utah, USA

****Correspondence:***
Pilar Cortés
mariapilar.cortes@uab.cat

† These authors have contributed equally to this work.

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INTRODUCTION

Non-typhoid *Salmonella* is the leading reported pathogen related to food-borne diseases, both in the European Union (EU) (European Food Safety Authority and European Centre for Disease Prevention Control, 2014) and in the USA (CDC, 2011). Salmonellosis in humans is often related to the ingestion of contaminated animal products (poultry, swine, beef, etc.) or of fruits and vegetables contaminated by animal waste (European Food Safety Authority and European Centre for Disease Prevention Control, 2014), consistent with the prevalence of certain serovars of *Salmonella enterica* in farm animals (e.g., Typhimurium and Enteritidis). The widespread antibiotic resistance in bacteria from various sources has had adverse effects on human health and has therefore encouraged the search for alternative antimicrobial agents (Endersen et al., 2014).

The natural biotherapeutic potential of bacteriophages is well recognized. Since 2006, different bacteriophage products have been assayed for use as therapeutics and food safety agents (Sulakvelidze, 2011). Bacteriophages and their derivatives are promising resources for use at each stage of the farm-to-fork. Recently, it has been reviewed their use for controlling of several major and emerging food-borne pathogens in both preharvest (farm animals) and postharvest (meat, fresh, and packaged foods) environments (Goodridge and Bisha, 2011). These studies reinforce the commercially exploiting of bacteriophages to diminish the economic weight of microbial contamination in foods and food processing environments.

To date, there is no evidence that bacteriophages exhibit harmful effects on humans or animals (Abedon et al., 2011). They are the most abundant entities and are present in all environments where a suitable host is found due to their high degree of host specificity (Kropinski et al., 2007). Nowadays, a security measure in the use of bacteriophages as antibacterials is that they must undergo whole-genome sequencing to ensure that the genome is free of genes encoding known bacterial virulence factors and potential immunoreactive allergens. Moreover, sequencing helps to understand the multiplicative cycle of bacteriophages at molecular level, and also other important biological traits. With this aim, the present work reports the sequencing and detailed analysis of the genomes of three *Salmonella*-specific bacteriophages (UAB_Phi20, UAB_Phi78, and UAB_Phi87) and the identification of the type of their genome ends. All three bacteriophages are able to infect not only a broad range of different strains of *S.* Typhimurium and *S.* Enteritidis serovars but also strains of the serovars Virchow, Hadar, and Infantis. They were previously selected from a collection of 55 bacteriophages isolated in poultry and pig feces obtained at different farms in Spain (Cortés et al., 2015). These bacteriophages are efficient against *S.* Typhimurium, both in poultry (Bardina et al., 2012; Colom et al., 2015) and in different food matrices (Spricigo et al., 2013).

MATERIALS AND METHODS

Bacteriophages

The three bacteriophages studied in this work, UAB_Phi20, UAB_Phi78, and UAB_Phi87, belong to a collection of 55 bacteriophages previously obtained from 189 chicken cloacae and pig rectal swabs collected from farms in different geographical areas of Spain between 2007 and 2009 (Cortés et al., 2015).

Bacteriophage DNA Extraction

High-titer (10^{11}–10^{12} pfu/ml in $MgSO_4$ 10 mM) lysates were obtained from each bacteriophage propagated in *S.* Typhimurium LB5000 strain (SGSC181; University of Calgary) and by ultracentrifugation at 51,000 × g for 2 h (Optima^{TM} L-80; Beckman, CA, USA) (Sambrook et al., 1989). Bacteriophage DNA was isolated using a phenol-chloroform method (Sambrook et al., 1989) with slight modifications. Phage suspensions were treated with DNase I (80 U/ml; Roche Diagnostics GmbH, Germany) and RNase I (80 μg/ml; Roche Diagnostics GmbH, Germany) at 37°C for 2 h. Following the addition of 0.5% sodium dodecyl sulfate (SDS, Sigma-Aldrich, St. Louis, MO, USA) and 200 μg proteinase K (Roche Diagnostics GmbH, Germany)/ml, they were incubated at 56°C for 2 h. Phage DNA was then extracted using phenol:chloroform and precipitated with ethanol. DNA integrity was checked by using a 0.7% agarose gel electrophoresis stained with Red Safe 1X (Intron Biotechnology; Seongnam-Si, Korea); the concentration was determined in a NanoDrop ND 1000 instrument (Thermo Scientific, DE, USA).

Bacteriophage DNA Sequencing and Genomic Analysis

The genomes of UAB_Phi20 and UAB_Phi78 were sequenced using the shotgun-full sequencing strategy. The UAB_Phi87 genome was sequenced using the Roche GS FLX system. All sequencing and sequence assembly procedures were done at Sistemas Genómicos (Valencia, Spain).

DNA sequences were analyzed using the software package DNAStar (DNAStar Inc.) and the online databases: http://www.ncbi.nlm.nih, http://www.ebi.ac.uk/, and http://cmr.jcvi.org. The whole-genome sequences of bacteriophages UAB_Phi20, UAB_Phi78, and UAB_Phi87 were deposited at GenBank under accession numbers GQ422450, GU595417, and JN225449, respectively. Possible open reading frames (ORFs) were predicted using the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). ORFs > 25 amino acids in length were further analyzed. Putative functions of ORFs were identified using the alignment search tools (BLASTP, BLASTX, and BLASTN search) of the National Center for Biotechnology Information (NCBI). ATG, GTG, and TTG were considered as start codons and TAA, TGA, and TAG as stop codons. Potential promoter regions and transcription terminators were predicted using the Softberry programs BProm (http://linux1.softberry.com/berry.phtml), FindTerm (Solovyev and Salamov, 2011), and TransTerm (Ermolaeva et al., 2000). The presence of a putative Shine-Dalgarno sequence (ribosome binding site, RBS) was confirmed based on its similarity to the *Escherichia coli* consensus sequence GGAGGT (Shine and Dalgarno, 1974). The tRNAscan-SE 1.21 program was used to search putative tRNAs (Lowe and Eddy, 1997). BLASTX and BLASTP were used to search for similarities with proteins in the database (Altschul et al., 1990). MAUVE (Darling et al., 2010) or ClustalW2 (McWilliam et al., 2013) were used for genome comparisons at the nucleotide level based on the genomic sequences available at NCBI (www.ncbi.

nlm.nih.gov). Comparisons at the proteomic level were made using CoreGenes (Turner et al., 2013). Phylogenetic analysis of phage large terminase subunit sequences was performed by using the ClustalW program in MEGA6 (Tamura et al., 2013). The tree based on neighbor-joining method was generated from a multiple alignment (gap opening penalty, 10; gap extension penalty, 1; and gap separation distance, 0). In order to obtain the tree, the parameters were set as following: (i) the model/method was set as number of differences; (ii) gaps/missing data treatment was established as complete deletion, and (iii) the random number generator seed and bootstrap trails were set at 111 and 1000, respectively (Casjens et al., 2005). Finally, the condensed tree was displayed with a bootstrap cut-off value of 70%.

Determination of the Bacteriophage Genome Ends

To identify potential *cos* ends, purified DNA of the three phages was digested with *Eco*RV restriction endonuclease (New England Biolabs, Hitchin, UK) at 37°C for 14 h. Two aliquots were then prepared. One was incubated at 60°C for 10 min to separate potentially ligated *cos* sites and immediately stored on ice. Restriction fragments length polymorphism patterns of heated and non heated aliquots were visualized by agarose gel electrophoresis (0.8%). Lambda bacteriophage DNA (Roche Diagnostics GmbH, Germany) with cohesive ends and treated with the same methodology served as a control.

On the other hand, the DNA from UAB_Phi20 was digested with *Eco*RI enzyme (New England Biolabs, Hitchin, UK) to detect an under-represented fragment as indicative of circularly permuted direct terminal repeats (DTR) in the chromosome ends. DNA of P22 bacteriophage was used as a control. Finally, to determine if the chromosome ends of UAB_Phi78 and UAB_Phi87 bacteriophages contain DTR, their DNA was treated with exonuclease *Bal*31, as described elsewhere (Klumpp et al., 2008). Briefly, 30 μg of bacteriophage DNA was treated with *Bal*31 nuclease (Takara; Saint-Germain-en-Laye, France) (0.5 units/μg) at 30°C for different incubation times. The reaction was stopped by the addition of 10 μl of EDTA (20 mM) followed by heating at 65°C for 5 min. DNA was purified using phenol-chloroform extraction and ethanol precipitation (Sambrook et al., 1989). Purified DNA (1 μg) was digested with *Hin*dIII (UAB_Phi78) and *Spe*I (UAB_Phi87) restriction enzymes (New England Biolabs, Hitchin, UK) and analyzed by agarose gel electrophoresis (1%). Those fragments that disappeared were newly isolated and purified (GE Healthcare Ltd., UK). *In silico* restriction of UAB_Phi78 and UAB_Phi87 with the adequate cutting sites were performed in order to identify the sequence of the disappeared fragments. In attention to these results, different primers were designed for sequencing the recovered and purified fragments. Finally, the sequences of DTR for both phages were confirmed by sequencing. To do this, the phage genomes were used as templates with primers that previously displayed drop-offs in the sequencing of the recovered fragments.

Isolation of UAB_Phi20 Lysogens

The possible lysogens present in the clear plaques of bacteriophage UAB_Phi20 on *S.* Typhimurium ATCC 14028 were picked from 10 plaques and streaked on green plates (Chan et al., 1972). Forty colonies were selected from these plates and streaked on green plates several times until they did not show dark green color. Overnight cultures in LB liquid medium of each colony were obtained and subcultured until an optical density at 550 nm (OD_{550}) of 1.0 was reached. Following, 0.5 μg/ml of mitomycin C (Sigma, St. Louis, MO) was added to the cultures and incubated at 37°C for 2 h. At that time, cultures were centrifuged and filtrated. A spotting assay of the supernatants with *S.* Typhimurium ATCC 14028 was conducted to ascertain the presence of induced UAB_Phi20. Similarly, a spotting assay of supernatants of overnight cultures was done.

RESULTS AND DISCUSSION

The adsorption kinetics and the lytic cycle of bacteriophages UAB_Phi20, UAB_Phi78, and UAB_Phi87 used as a cocktail in therapy strategies against *S.* Typhimurium (Bardina et al., 2012; Spricigo et al., 2013; Colom et al., 2015) were previously characterized. They exhibited similar adsorption constant (K) ranging between 1.1×10^{-9} and 1.2×10^{-9} ml cfu^{-1} min^{-1} and the timing of the latent period of bacteriophages UAB_Phi20, UAB_Phi78, and UAB_Phi87 was 46.0, 26.7, and 58.0 min, respectively (Bardina, 2011; Spricigo, 2011). The burst sizes of UAB_Phi20 and UAB_Phi78 were similar (95.0 and 87.7 pfu/cfu, respectively) while that of UAB_Phi87 was 55 pfu/cfu (Bardina, 2011; Spricigo, 2011). In addition, they were previously characterized with respect to broad host range, restriction patterns, RAPD profiles, morphology, genome size and lytic activity *in vitro* (Bardina et al., 2012; Cortés et al., 2015).

In this study, we report the whole genome sequencing and some traits of their biology at molecular level. *In silico* analyses of bacteriophage genomes did not show any similarities neither to known virulence-associated genes nor to any antibiotic resistance genes or potential immunoreactive food allergens (FARRP, 2011). It must be noted that a high percentage of hypothetical proteins were found in their genomes. This agrees with the reported for all sequenced bacteriophages which has been widely commented by the scientific community (Klumpp et al., 2013). Therefore, the identification of their function is a challenge that must be addressed for increasing the knowledge of the bacteriophages and the level of security of their applications. In this regard, it must be considered that none of hypothetical proteins showed significant similarity to known or hypothetical factors involved in bacterial pathogenicity. Therefore, it is unlikely that they have a role in bacterial virulence. Additionally, in our reported *in vivo* experiments (Bardina et al., 2012), we inoculated the phage cocktail, with and without their host, and no harmful signs were observed in animals. In attention to the above indicated, and given the large amount of information available in bacterial gene databases, the three phages studied are safe with respect to our current knowledge.

Genome Analysis of UAB_Phi20

The genome of UAB_Phi20 consisted of linear double-stranded DNA (ds DNA), 41,809 base pairs (bp) in length and with an overall genomic guanine plus cytosine (G+C) content of

47.2% which is slightly lower than its host (52.2%). ORF Finder revealed 80 possible ORFs. The annotation and organization of the UAB_Phi20 genome are provided in **Table 1**. Given the high level of genome compaction, many of the promoters identified in UAB_Phi20 overlapped in their coding regions. Therefore, potential promoters were sought using the BPROM program (Softberry), limiting the search to a maximum distance of 100 bp relative to the start of the potential UAB_Phi20 phage genes. All 12 hypothetical promoters thus identified (Table S1) had a highly conserved −10 consensus sequence (TATAAT), while in the −35 box (TTGACA) only the second T and the G were strongly conserved. In addition, there were 11 putative Rho-independent terminators (**Figure 1**). ATG was the start codon in all ORFs except gene *p80*, in which TTG was the start codon. The three stop codons were present in different proportions, with TAA as the most common (56.3% of the genes), followed by TGA and TAG (in 36.2 and 7.5% of the ORFs, respectively). The RBS Finder (Glimmer) program revealed partial conservation of the ribosome-binding sites (RBS) of bacteriophage UAB_Phi20 with respect to the Shine-Dalgarno consensus sequence (AGGAGG). Interestingly, the distance of this sequence from the translation initiation site was not conserved in all genes but instead ranged from only 7 to 40 bp.

The genomic annotation and the analysis of the genetic organization of phage UAB_Phi20 showed high homology with that of *Salmonella* bacteriophage P22 and other *P22likevirus*. Functions were assigned to 42 ORFs of the 80 identified (**Table 1**). In addition, 14 ORFs corresponded to *ea* and *nin* regions. Of the remaining 24 ORFs, 16 encoded proteins which showed similarity with hypothetical proteins already described, but their functions could not be determined, and 8 ORFs showed no similarity with any protein available in the databases. The proteins of UAB_Phi20 were classified into different functional groups (**Figure 1**).

The lysogeny group included proteins involved on the establishment of lysogeny, lysogenic conversion, immunity, the excisionase and the attP region. The establishment of lysogeny requires the activity of the integrase encoded by *int* gene. This protein showed an identity ≥98% compared to the counterparts in bacteriophages P22, ST64T, and ST104. A hypothetical *attP* site with a sequence similar to that described in P22 was also found between the genes *int* and *gtrA*. The products of the genes *c2*, *cro*, *c1*, and *c3* genes, which directly affect phage decision between lytic or lysogenic cycle, and those encoded by *mnt*, *arc,* and *ant* genes, which are involved in the control of the maintenance of lysogeny (Susskind and Botstein, 1978) showed a high similarity with those of P22. During lysogenic conversion, the lysogenization of bacterial cells with certain lambdoid bacteriophages produces a chemical change in the bacterial lipopolysaccharide O antigen such that the binding of other bacteriophages that recognize the same receptor is prevented (Kropinski et al., 2007). The UAB_Phi20 genes that are responsible for this function are *gtrC*, *gtrB*, and *gtrA*; all of their products showed ≥99% homology with their counterparts in bacteriophages P22, ST64T, and ST104. The genome of UAB_Phi20 also contains three genes (*17*, *sieA*, and *sieB*) encoding proteins involved in the exclusion of superinfection (immunity). These proteins were very similar to those of phage P22. Protein 17 participates in the release of exclusion by heterologous phages such as Fels-1, whereas SieB and SieA prevent infection by heteroimmune phages or superinfection by the own phage (Susskind and Botstein, 1978). In addition, the excisionase (*xis*) showed an identity of 100% compared to the corresponding protein in P22 and ST64T, whereas for ST104 the identity was ∼97%.

The gene products involved in the DNA metabolism of UAB_Phi20 were identical to those of ST104 but had only ∼70% identity with those of phage P22. This group of genes included *abc2* and *abc1*, encoding a protein with an anti-RecBCD function, and the hypothetical *erf* and *arf* genes, involved in the recombination and recircularization of phage DNA (Poteete et al., 1988). Genome replication by UAB_Phi20 requires two proteins similar to the helicase (Gp12) and primase (Gp18) proteins of P22 (Vander Byl and Kropinski, 2000).

A cluster of UAB_Phi20 genes involved in the bacterial lysis encoding holin (*gp13*), lysozyme (*gp19*), and two endopeptidases (*gp15* and *Rz1*) were identified. All these proteins were identical to those of phage P22. After the endopeptidases, the *orf21*, which may also play a role in bacterial lysis, was identified. However, as no gene homologous to *orf21* was found in the databases, neither its function nor its assignment to the lysis region could be confirmed.

Genes involved in structure and assembly could be divided into those encoding terminases, capsid, DNA injection, or tail proteins. The major part of proteins involved in these functions was similar to the respective proteins of phage P22 and presented a high identity with those encoded by bacteriophages ST104 and ST64T. For example, UAB_Phi20 tail-spike and the major capsid proteins were almost identical (≥99%) to the respective proteins of bacteriophages P22, ST104 and ST64T. Both the small and large terminases, encoded by *gp3* and *gp2*, respectively, had an identity of ∼100% with the genes of P22, ST104, and ST64T phages. In a recent work comparing 57 P22-like bacteriophages (Casjens and Thuman-Commike, 2011), terminases and capsid proteins were the most conserved, whereas the most divergent proteins were related to host recognition, such as tail and injection proteins. However, the high identity found by us for all these proteins indicates a low divergence and strong phylogenetic relationship between UAB_Phi20 and bacteriophages P22, ST104, and ST64T. In addition, the genome of UAB_Phi20 contained a unique site (*pac*) located within the sequence of the small subunit of terminase. *Pac* sequence (GAAGACTTATCTGAGGTCGTTA) differed by two bases from the corresponding sequence of P22 (Wu et al., 2002).

Besides regions above commented, other important feature of the UAB_Phi20 genome was the identification of *ea* and *nin* regions, which encoded a number of proteins of unknown function. Neither of these genes is essential for bacteriophage function, at least in *in vitro* cultures, but their presence and maintenance suggest that they confer a selective advantage for

TABLE 1 | Features of bacteriophage UAB_Phi20 genome, ORFs, gene products, and functional assignments.

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best *e*-value
		From	To						
1		27	173	+	48	Unknown	Hypothetical protein P22gp50(NP_059609.1)	100	9.E-25
2	*gp18*	166	981	+	271	DNA replication (primase)	Hypothetical protein P22gp51 (NP_059610.1)	100	0
3	*gp12*	978	2354	+	458	DNA replication (helicase)	Hypothetical protein P22gp52 (NP_059611.1)	100	0
4	*ninA*	2351	2431	+	26	Unknown	Nin A [Enterobacteria phage P22](YP_063725.1)	100	5.E-20
5	*ninB*	2428	2865	+	145	Unknown	NinB [Enterobacteria phage P22] (NP_059612.1)	99	6.E-101
6	*ninD*	2862	3035	+	57	Unknown	Nin D [Enterobacteria phage P22] (YP_063726.1)	100	7.E-35
7	*ninE*	3002	3178	+	58	Unknown	Nin E [Enterobacteria phage P22] (NP_059614.1)	100	6.E-34
8	*ninX*	3175	3513	+	112	Unknown	Nin X [Enterobacteria phage P22] (NP_059615.1)	100	1.E-77
9	*ninF*	3506	3682	+	58	Unknown	Nin F [Enterobacteria phage P22] (YP_063727.1)	100	2.E-33
10	*ninG*	3672	4286	+	203	Unknown	Nin G [Enterobacteria phage P22] (YP_063728.1)	100	6.E-145
11	*ninY*	4283	4507	+	74	Unknown	Nin Y [Enterobacteria phage P22] (NP_059618.1)	100	5.E-48
12	*ninH*	4504	4707	+	67	Unknown	Nin H [Enterobacteria phage P22] (NP_059619.1)	100	3.E-41
13	*ninZ*	4688	4867	+	59	Unknown	Nin Z [Enterobacteria phage P22] (YP_063729.1)	100	3.E-34
14	*23*	4864	5487	+	207	Transcription antitermination protein	Gp63 [Enterobacteria phage P22] (YP_063730.1)	100	9.E-153
15		5577	5786	+	69	Unknown	Hypothetical protein ε34gp63 (YP_002533523.1)	97	2.E-40
16		5809	5913	+	34	Unknown			
17	*gp13*	5922	6248	+	108	Holin	Holin [Enterobacteria phage P22] (NP_059621.1)	100	1.E-70
18	*gp19*	6229	6669	+	146	Lysozyme	Gp66 [Enterobacteria phage P22] (NP_059622.1)	100	1.E-101
19	*gp15*	6804	7103	+	99	Endopeptidase Rz	Hypothetical protein P22gp67 (NP_059623.2)	100	2.E-64
20	*Rz1*	6838	7050	+	70	Lipoprotein Rz1 precursor	Hypothetical protein P22gp68 (YP_063732.1)	100	1.E-34
21		7144	7329	+	61	Unknown			
22	*Rha*	7322	7858	+	178	Unknown	Rha [Enterobacteria phage P22] (NP_059624.1)	100	9.E-130
23		7940	8296	+	118	Unknown	Hypothetical protein SE1gp48 (YP_002455884)	100	7.E-78
24		8300	8689	+	129	Unknown	Hypothetical protein ST64Tp49 (NP_720323.1)	100	8.E-92
25		8689	9093	+	134	Unknown	Hypothetical protein ST64Tp50 (NP_720324.1)	100	2.E-90
26	*gp3*	9097	9585	+	162	Terminase (small subunit)	ST64Tp51 (NP_720325.1)	100	3.E-116
27	*gp2*	9737	11062	+	441	Terminase (large subunit)	Gp2 [Enterobacteria phage ST104] (YP_006405)	99	0
28	*gp1*	11062	13239	+	725	Portal protein	Gp1 [*Salmonella enterica* bacteriophage SE1] (YP_002455889.1)	100	0

(Continued)

TABLE 1 | Continued

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best *e*-value
		From	To						
29	*gp8*	13253	14164	+	303	Scaffolding protein	Gp8 [Enterobacteria phage P22] (YP_063736.1)	100	0
30	*gp5*	14164	15456	+	430	Coat protein	Coat protein [Enterobacteria phage ST64T] (NP_720329.1)	99	0
31		15495	15704	+	69	Unknown	Hypothetical protein P22gp06 (NP_059631.1)	100	5.E-40
32	*gp4*	15688	16188	+	166	DNA stabilization protein	Gp4[Enterobacteria phage P22] (NP_059632.1)	100	8.E-120
33	*gp10*	16148	17566	+	472	Packaged DNA stabilization protein	Head completion protein [Enterobacteria phage P22] (NP_059633.1)	100	0
34	*gp26*	17570	18271	+	233	Head completion protein	Gp26[Enterobacteria phage P22] (YP_063715.1)	100	5.E-165
35	*gp14*	18271	18726	+	151	Head assembly protein	Gp14 [Enterobacteria phage P22] (YP_063716.1)	100	6.E-109
36	*gp7*	18729	19418	+	229	Injection protein	Gp7 [Enterobacteria phage P22] (YP_063717.1)	100	2.E-154
37	*gp20*	19429	20844	+	471	Injection protein	Gp20 [Enterobacteria phage P22] (NP_059637.1)	100	0
38	*gp16*	20844	22673	+	609	Injection protein	Gp16 [Enterobacteria phage P22] (YP_063718.1)	100	0
39	*sieA*	23406	22696	–	236	Superinfection exclusion	SieA [Enterobacteria phage P22] (NP_059639.1)	99	7.E-110
40	*hkcC*	23221	23586	+	121	Unknown	hkcC [Bacteriophage HK620] (NP_112089.1)	100	5.E-85
41		23794	23600	–	64	Unknown	Hypothetical protein P22gp16 (NP_059640.1)	98	5.E-33
42	*mnt*	24130	23879	–	83	Maintenance of lysogeny	Mnt [Enterobacteria phage P22] (NP_059641.1)	100	2.E-53
43	*arc*	24158	24382	+	74	Transcriptional repressor	Repressor arc [Escherichia coli MS 16-3] (EFU59036.1)	99	8.E-46
44	*ant*	24451	25353	+	300	Antirepressor	Ant [Enterobacteria phage P22] (NP_059643.1)	100	0
45	*gp9*	25564	27567	+	667	Tailspike protein (Endorhamnosidase)	Tailspike protein [Enterobacteria phage P22] (AAF75060.1)	99	0
46	*gtrC*	28858	27626	–	410	O-antigen conversion; glucosyl transferase	GtrC [Enterobacteria phage P22] (YP_063719.1)	100	0
47		28879	28983	–	34	Unknown			
48	*gtrB*	30050	29073	–	325	O-antigen conversión; bactoprenol glucosyl transferase	GtrB [Enterobacteria phage ST64T] (NP_720276.1)	99	0
49	*gtrA*	30364	30002	–	120	O-antigen conversión; translocase (flipase)	GtrA [Enterobacteria phage P22] (NP_059583.1)	100	2.E-80
50		30538	30660	+	40	Unknown			
51	*int*	31876	30713	–	387	Integrase	Int [Enterobacteria phage P22] (NP_059584.1)	99	0
52	*xis*	32103	31753	–	116	Excisionase	Xis [Enterobacteria phage P22] (NP_059585.1)	100	3.E-79
53	*eaC*	32741	32106	–	211	Unknown	EaC [Enterobacteria phage P22] YP_063720.1	99	7.E-154
54	*eaG*	33021	32842	–	60	Unknown	EaG [Enterobacteria phage P22] (NP_059587.1)	100	4.E-34
55	*eaA*	34071	33118	–	317	Unknown	EaA [Enterobacteria phage P22] (NP_059588.1)	100	0

(Continued)

TABLE 1 | Continued

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best *e*-value
		From	To						
56	*eal*	34269	34075	–	64	Unknown	Eal [Enterobacteria phage P22] (NP_059589.1)	100	3.E-36
57		35147	34266	–	293	Unknown	ORF8 [Enterobacteria phage ST104] (YP_006364.1)	91	1.E-116
58		35726	35217	–	169	Unknown	ORF9 [Enterobacteria phage ST104] (YP_006365.1)	100	2.E-118
59		35893	35723	–	56	Unknown	ORF10 [Enterobacteria phage ST104] (YP_006366.1)	100	7.E-33
60	*abc2*	36197	35904	–	97	Anti Rec-BCD protein	Abc2 [Enterobacteria phage ST104] (YP_006367.1)	100	4.E-63
61	*abc1*	36528	36244	–	94	Anti Rec-BCD protein	Abc1 [Enterobacteria phage ST104] (YP_006368.1)	100	3.E-62
62	*erf*	37235	36528	–	235	Recombination protein	ORF13 [Enterobacteria phage ST104] (YP_006369.1)	100	1.E-173
63	*arf*	37375	37232	–	47	Recombination protein	Arf [Enterobacteria phage P22] (NP_059597.1)	100	3.E-24
64	*kil*	37553	37365	–	62	Inhibitor of host septation	Kil [Enterobacteria phage P22] (NP_059598.1)	100	2.E-38
65	*c3*	37692	37534	–	52	Regulatory protein	C3 [Enterobacteria phage P22] (NP_059599.1)	100	2.E-29
66	*17*	38089	37778	–	103	Superinfection exclusion	Hypothetical protein P22gp40 (NP_059600.1)	100	9.E-70
67		38040	38159	+	39	Unknown			
68		38440	38237	–	67	Unknown	Hypothetical protein P22gp41 (CAA33649.1)	99	4.E-41
69		38676	38440	–	78	Unknown	Hypothetical protein P22gp42 (NP_059602.1)	100	2.E-47
70		38773	38648	–	41	Unknown	Hypothetical protein ST64Tp22 (NP_720296.1)	92	5.E-07
71	*ral*	38907	38713	–	64	Antirestriction protein	Ral [Enterobacteria phage P22] (NP_059603.1)	100	2.E-37
72		38977	38891	–	28	Unknown	Hypothetical protein lambdap47 (NP_040623.1)	100	4.E-27
73	*sieB*	38945	39700	+	251	Superinfection exclusion	SieB [Enterobacteria phage P22] (NP_059604.1)	100	3.E-138
74	*24*	40023	39721	–	100	Antitermination protein	Hypothetical protein P22gp46 (NP_059605.1)	100	3.E-65
75		40043	40255	+	70	Unknown			
76	*c2*	41027	40377	+	216	Prophage repressor	C2 [Enterobacteria phage P22] (NP_059606.1)	100	4.E-158
77	*cro*	41108	41293	+	61	Antirepressor	Cro [Enterobacteria phage P22] (NP_059607.1)	100	2.E-35
78		41354	41241	–	37	Unknown			
79	*c1*	41400	41678	+	92	Transcriptional activator	C1 [Enterobacteria phage P22] (NP_059608.1)	100	2.E-59
80		41668	41809	+	46	Unknown			

Gene numbers correspond with their predicted function, if known, followed by the nature of the evidence that supports the functional classification. Genes with no functional prediction, but with significant sequence similarity to genes in the NCBI database as determined by BLASTP are also listed.

either the host or the bacteriophage itself when present in other environments (Hendrix, 2002).

Although the UAB_Phi20 genome contains all the elements for giving a lysogenic cycle, infected-*Salmonella* cultures by this bacteriophage were completely cleared and UAB_Phi20 plaques were also typically clear. Both observations suggested that this phage is virulent and unable to promote a lysogenic cycle. The possible reasons of this apparent contradiction were studied. First of all we considered that the hypothetical *attP* sequence of UAB_Phi20 is similar to that described in P22 bacteriophage.

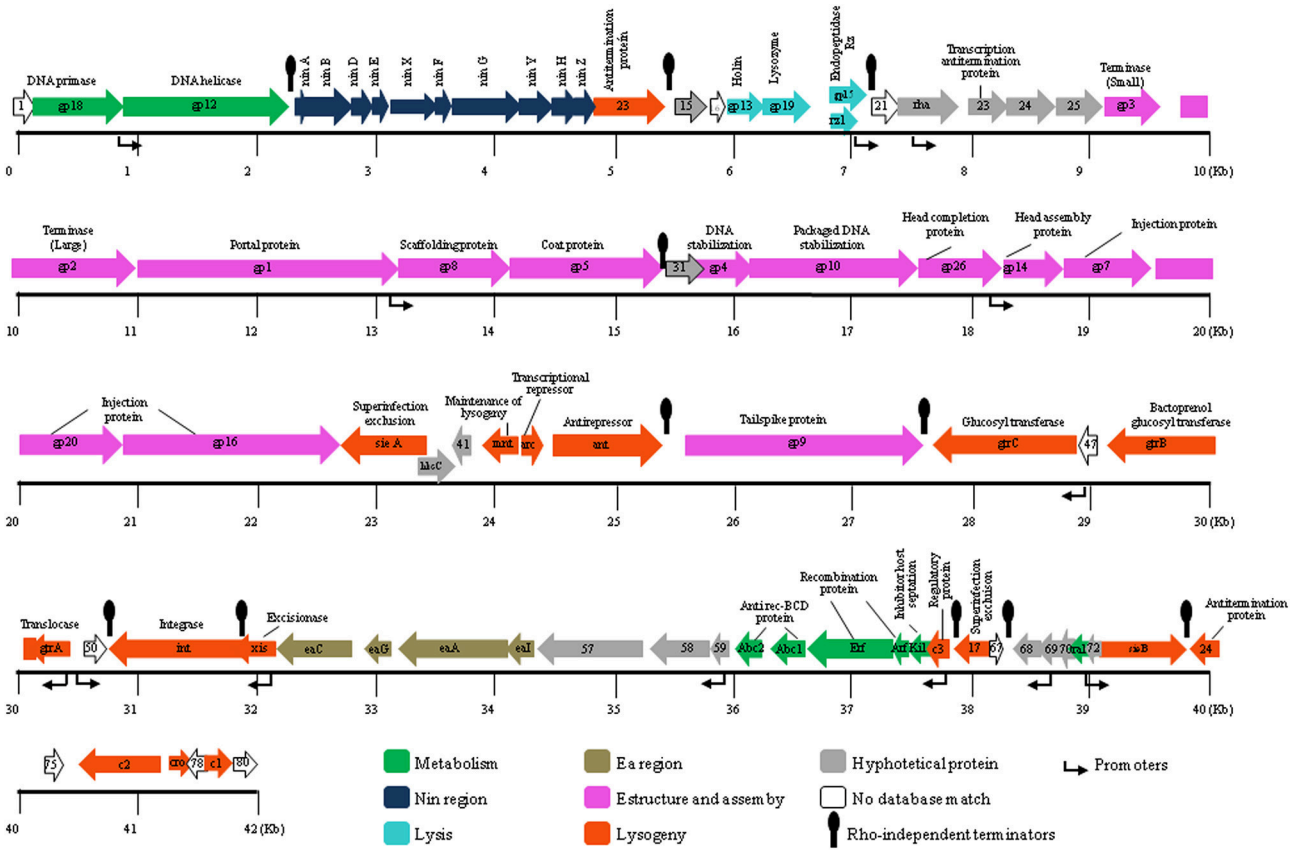

FIGURE 1 | Genomic structure of bacteriophage UAB_Phi20 including the Rho-indepedent terminators. Arrows represent genes, and the different colors identify the functional category into which the homologous genes were classified. Gene functions are indicated where they are known. The color code for gene function is provided at the bottom of the figure. ORFs are numbered consecutively from left to right as described in **Table 1**, and are indicated by arrows pointing to the direction of transcription.

Therefore, bacteriophage UAB_Phi20 could integrate into the P22 site (*attB*) of the *Salmonella* chromosome (Smith-Mungo et al., 1994). However, we were unable to detect this by PCR amplification studies (data not shown). In addition, the possible C1 recognition motif (TTGN6TTGC) in the UAB_Phi20 genome was not identified neither at the region of the P_{RE} promoter nor in the vicinity of the gene encoding integrase and, as consequence, the repressor of the lytic cycle cannot be transcribed. However, these data did not discard that UAB_Phi20 had a very low frequency of lysogenization which could result in apparently clear plaques. To test this, the possible lysogens present in the clear plaques were picked and streaked on green plates. Afterwards, 40 colonies were selected from these plates and, for removing the possible bacteriophages coming from plaques, they were streaked on green plates until they did not show dark green color. If UAB_Phi20 had a low frequency of lysogenization, it would be expected that some of these colonies were stable lysogens. However, the treatment of liquid cultures of those colonies with mitomycin C did not yield bacteriophage production. In addition, no bacteriophages were detected in the supernatant of overnight cultures of these colonies. All these results evidenced that the bacteriophage UAB_Phi20 is unable to give rise a lysogenic cycle producing stable lysogens on this host.

Comparison of the genome of UAB_Phi20 with those of P22, ST64T, and ST104 at protein level using CoreGenes (Turner et al., 2013) revealed that shared 72% of its proteins with P22 and 63–65% with those of ST64T, and ST104. These results agree with that obtained by BlastP with protein-by-protein comparison (**Table 1**) and allow classifying UAB_Phi20 into *P22likevirus* genus as sharing at least 40% of proteins is a requisite to be classified into a determined genus (Lavigne et al., 2008). Finally, alignment of the annotated genomes of these bacteriophages using Mauve demonstrated the considerable sequence similarity between UAB_Phi20 and P22. Few noticeable differences with respect to ST64T and ST104 bacteriophages, especially at region 34–40 kb on the UAB_Phi20 genome, were observed (**Figure 2**). The high similarities between their genes, their organization and the identification of hypothetical genes lacking similarity with the above-mentioned bacteriophages demonstrate the genome mosaicism of these members of the *Podoviridae*. The origin of this genetic mosaicism agrees with the model of modular evolution of bacteriophages in which the horizontal transfer of genetic modules and their incorporation by

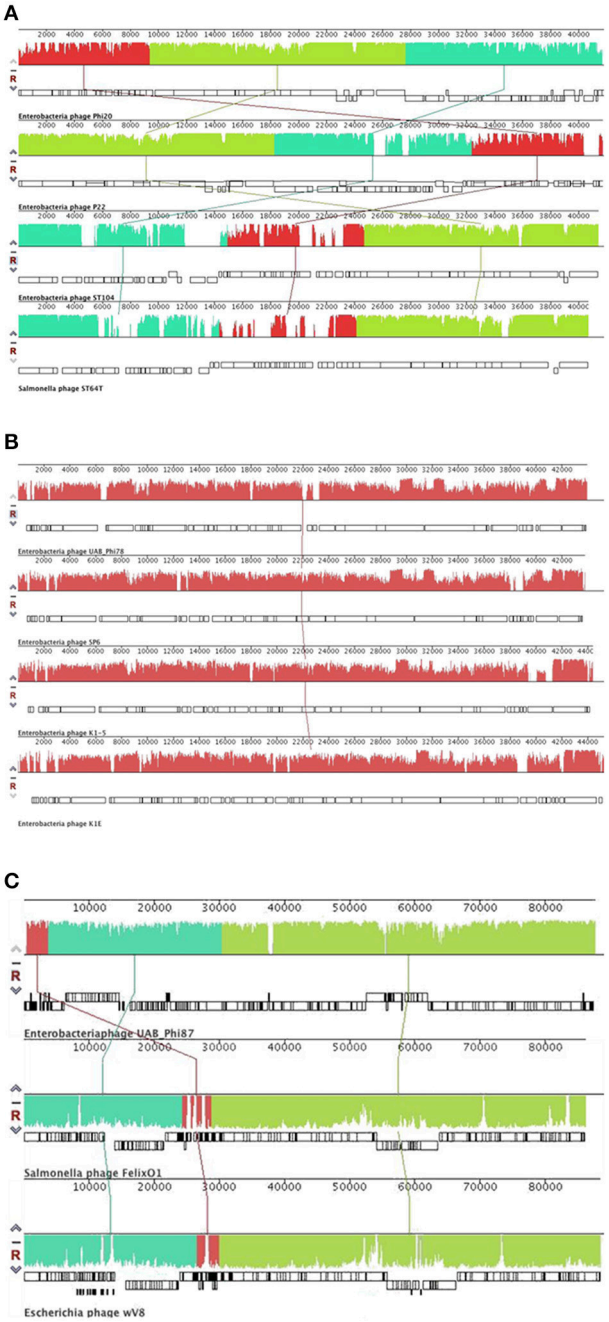

FIGURE 2 | Alignment of the UAB_Phi20 (A), UAB_Phi78 (B), and UAB_Phi87 (C) genomes and their counterparts belonging to the *Podoviridae* and *Myoviridae* families using Progressive MAUVE. The names of the different bacteriophages are indicated under their maps. Colored blocks correspond to regions of nucleotide similarity which is indicated by its height and regions with a lack of homology are outside these blocks or indicated in white inside the blocks. The tRNA genes are indicated by the filled gene blocks.

homologous recombination leads to new genetic combinations that give rise to new lambdoid bacteriophages (Thomson et al., 2004).

Genome Analysis of UAB_Phi78

The UAB_Phi78 genome is a linear dsDNA molecule of 44,110 bp including DTR of 179 bp and with a G+C content of 47.41%, slightly lower than that of *Salmonella* (52.2%). Genome analysis predicted 58 putative ORFs (**Table 2**, **Figure 3**). The genome annotation of the SP6 bacteriophage (Genbank accession number NC_004831) was used to assign similarities to UAB_Phi78 ORFs because the genome of UAB_Phi78 showed the highest similarity (86%) with the genome of this phage after analysis with ClustalW2 program.

A BPROM search identified 25 promoters (Table S1). Each had a −10 and −35 consensus sequences, ggTAtaaT and TTGAca, respectively (the conserved bases are indicated in capital letters). Four potential Rho-factor independent terminators were also identified in the UAB_Phi78 genome with FindTerm program (**Figure 3**). The first was located after gene encoding the RNA polymerase (*gp8*); the second and fourth immediately after genes encoding a protein of unknown function, and the third downstream of the gene (*gp32*) encoding the major capsid protein. Additionally, a fifth terminator was identified with the Transterm program. This was located after the genes encoding the tail spike protein. For 57 of the 58 predicted genes ATG was the translation initiation codon; in the remaining gene, *orf39*, the start codon was TTG. TAA was the most prevalent (67.2%) stop codon, followed by TGA and TAG (19 and 13.8%, respectively).

Among the 58 ORFs, 20 could be assigned functions and showed significant similarity with reported proteins of the SP6 bacteriophage (Dobbins et al., 2004). Hypothetical proteins were encoded by 26 ORFs whereas 12 did not show similarity with any gene product of the databases. According to a homology-search-based annotation the ORFs of UAB_Phi78 were categorized into three functions. Within the metabolic functions, the protein encoded by *orf20* showed significant identity (95%) with the DNA polymerase encoded by gene SP6 *gp14*, suggested to be the origin of bidirectional replication in SP6 (Dobbins et al., 2004). Likewise, proteins associated with the DNA metabolism of the phage genome were also identified: RNA polymerase (Gp8), DNA primase (Gp10), exonuclease (Gp21), endonuclease (Gp22), and DNA ligase (Gp25). All of them showed an identity of ∼95% with their counterparts in the SP6 genome (Dobbins et al., 2004). It is remarkable that this phage encodes a RNA polymerase that may control the expression of its own DNA polymerase, similar to that described for the phage T7 (Kropinski et al., 2007). This could promote an efficient transcription of UAB_Phi78 genes and justify that the timing of the latent period was significantly lower than that of the other two phages studied in this work (Bardina, 2011; Spricigo, 2011). Accordingly, by PCR amplification, the UAB_Phi78 DNA was detected 10 min after infection of bacterial cells, whereas the UAB_Phi20 and UAB_Phi87 DNA were seen 20 min after infection (data not shown).

Finally, it is noteworthy that protein encoded by *orf6* of the UAB_Phi78 bacteriophage has 75% identity with SP6 Gp5 protein which encodes a putative anti-restriction protein. It has been suggested that it is the responsible for phage multiplication in *Salmonella* cells with or without its natural type I restriction systems (Scholl et al., 2004). Obviously, this can confer an advantage over other bacteriophages.

TABLE 2 | Features of bacteriophage UAB_Phi78 genome, ORFs, gene products, and functional assignments.

ORF	Gene	Position (nt)		No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best *e*-value
		From	To					
1		662	949	95	Unknown			
2		954	1016	20	Unknown			
3		1062	1241	59	Unknown			
4		1238	1417	59	Unknown	Gp3 Bacteriophage SP6 (AAP48742.1)	88	4.E-28
5		1410	1619	69	Unknown	Gp4 [Bacteriophage SP6] (AAP48743.1)	76	5.E-25
6		1773	2129	118	Unknown	Gp5 [Bacteriophage SP6] (AAP48744.1)	75	3.E-36
7		2130	2249	39	Unknown			
8		2319	2474	51	Unknown			
9		2537	3412	291	Unknown	Gp6 [Bacteriophage K1E] (CAJ29406.1)	72	2.E-142
10	*gp8*	3487	6111	874	DNA-directed RNA polymerase	Gp8 [Bacteriophage SP6] (AAP48747.1)	98	0
11		6771	6845	24	Unknown			
12		6849	6974	41	Unknown	gene 1.1 [Bacteriophage T7] (AAP33970.1)	65	0.012
13	*gp10*	6976	8871	631	DNA primase	Gp10 [Bacteriophage SP6] (AF159357.1)	98	0
14		9065	9469	134	Unknown			
15		9381	9614	77	Unknown			
16		9607	9807	66	Unknown	Gp11 [Bacteropphage SP6] (AAP48750.1)	89	2.E-34
17		9758	10000	80	Unknown	Gp12 [Bacteriophage SP6] (AAP48751.1)	93	9.E-27
18		10068	10172	33	Unknown	Gp11.5 [Bacteriophage K1E] (CAJ29415.1)	59	0.004
19		10159	10377	72	Unknown	Gp12 [Bacteriophage K1E] (CAJ29416.1)	93	3.E-42
20	*gp14*	10364	12910	848	DNA polymerase	Gp14 [Bacteriophage SP6] (AAP48753.1)	95	0
21		12910	13008	32	Unknown	Gp15 [Bacteriophage SP6] (AAP48754.1)	75	6.E-07
22		13123	13500	125	Unknown	Gp17 [Bacteriophage SP6] (AAP48756.1)	64	2.E-47
23		13580	14389	269	Unknown	Gp18 [Bacteriophage SP6] (AAP48757.1)	92	6.E-180
24		14407	14625	72	Unknown	Gp19 [Bacteriophage SP6] (AAP48758.1)	100	4.E-45
25		14645	14728	27	Unknown			
26		14731	15099	122	Unknown	Gp20 [Bacteriophage SP6] (AAP48759.1)	92	1.E-62
27		15165	15476	103	Unknown			
28	*gp21*	15383	16414	343	Exonuclease	Gp21 [Bacteriophage SP6] (AAP48760.1)	96	0
29	*gp22*	16399	16809	136	Endonuclease	Gp22 [Bacteriophage SP6] (AAP48761.1)	97	4.E-90
30		16895	17809	304	Unknown	Gp23 [Bacteriophage SP6] (AAP48762.2)	98	0
31		17910	18359	149	Unknown	23 [Bacteriophage K1-5] (AAR90065.1)	63	4.E-53
32	*gp25*	18359	19306	315	DNA ligase	Gp25 [Bacteriophage SP6] (AAP48764.1)	96	0
33		19278	19493	71	Unknown	Gp26 [Bacteriophage SP6] (AAP48765.1)	98	6.E-35

(Continued)

TABLE 2 | Continued

ORF	Gene	Position (nt)		No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best *e*-value
		From	To					
34		19456	19599	47	Unknown	Gp27 [Bacteriophage SP6] (AAP48766.1)	90	1.E-09
35		19629	20090	153	Unknown	Gp28 [Bacteriophage SP6] (AAP48767.1)	98	6.E-106
36		20100	20309	69	Unknown	Gp29 [Bacteriophage SP6] (AAP48768.1)	99	9.E-38
37	*gp30*	20311	21858	515	Portal protein	Gp30 [Bacteriophage SP6] (AAP48769.1)	99	0
38	*gp31*	22329	22706	125	Scaffolding protein	Gp31 [Bacteriophage SP6] (AAP48770.1)	92	2.E-71
39		22782	23078	98	Unknown			
40	*gp32*	23256	24458	400	Major capside protein	Gp32 [Bacteriophage SP6] (AAP48771.1)	98	0
41	*gp33*	24514	25254	246	Tail protein	Gp33 [Bacteriophage SP6] (AAP48772.1)	98	0
42	*gp34*	25254	27677	807	Tail protein	Gp34 [Bacteriophage SP6] (AAP48773.1)	98	0
43	*gp35*	27668	28387	239	Internal virion protein	Gp35 [Bacteriophage SP6] (AAP48774.1)	98	1.E-165
44		28388	31324	978	Unknown	Gp36 [Bacteriophage SP6] (AAP48775.1)	99	0
45	*gp37*	31391	35203	1270	Internal virion protein	Gp37 [Bacteriophage SP6] (AAP48776.1)	99	0
46	*gp38*	35203	36162	319	Tail fiber protein (adaptor)	Gp38 [Bacteriophage SP6] (AAP48777.1)	98	0
47	*gp39*	36171	36365	64	Putative holin	Gp39 [Bacteriophage SP6] (AAP48778.1)	97	2.E-35
48	*gp40*	36505	36651	48	Terminase (small subunit)	Gp40 [Bacteriophage SP6] (AAP48779.1)	96	4.E-23
49	*gp41*	36651	38549	632	Terminase (large subunit)	Gp41 [Bacteriophage SP6] (AAP48780.1)	97	0
50		38702	38977	91	Unknown	Gp41 [Bacteriophage K1E] (CAJ29452.1)	76	2.E-40
51	*gp42*	38993	39280	95	Putative Acetyl-CoA acetyltransferase	Gp42 [Bacteriophage K1E] (CAJ29453.1)	83	8.E-47
52	*gp46*	39283	39627	114	Peptidase_M15_3	Gp46 [Bacteriophage SP6] (AAP48785.1)	75	4.E-60
53		39627	39764	45	Unknown			
54		39981	40166	61	Unknown	Gp45 [Bacteriophage K1E] (CAJ29456.1)	88	6.E-30
55	*gp49*	40297	41949	550	Tail spike protein	Gp49 [Bacteriophage SP6] (AAP48788.1)	98	0
56		42033	43547	504	Unknown	Gp50 [Bacteriophage SP6] (AAP48789.1)	96	0
57		43555	43698	47	Unknown	Gp51 [Bacteriophage SP6] (AAP48790.1)	96	3.E-22
58		43752	43829	25	Unknown	Gp52 [Bacteriophage SP6] (AAP48791.2)	100	3.E-19

Gene numbers correspond with their predicted function, if known, followed by the nature of the evidence that supports the functional classification. Genes with no functional prediction, but with significant sequence similarity to genes in the NCBI database as determined by BLASTP are also listed.

With respect to lysis, the *orf47* encoded a protein with a 96% identity with a putative holin codified in the SP6 *gp39* gene. Similar to the results in SP6, K1E, and K1–5 bacteriophages, no endolysin homologous to that encoding T7*gp18.5* and involved in lysis was identified (Dobbins et al., 2004; Scholl et al., 2004). This gene has only been identified in the genome of bacteriophage K1F

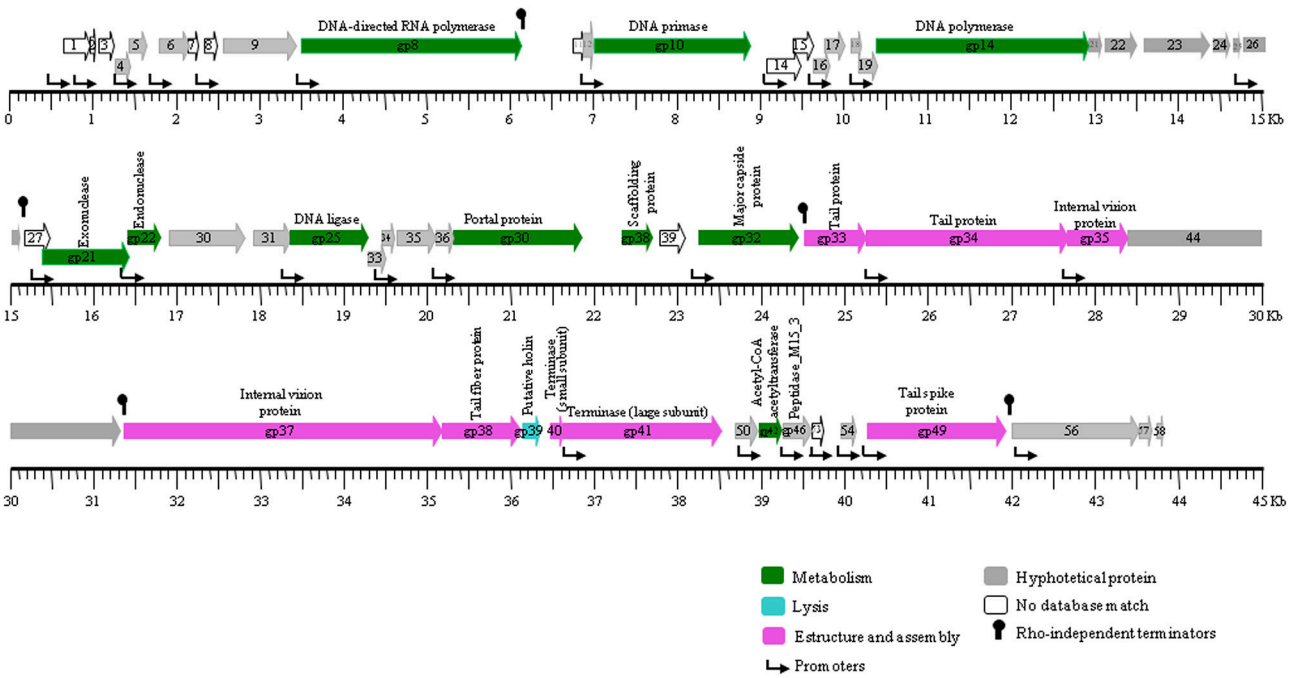

FIGURE 3 | Genomic structure of UAB_Phi78 including the Rho-indepedent terminators. Arrows represent genes, and the different colors identify the functional category into which the homologous genes were classified. Gene functions are indicated where they are known. The color code for gene function is provided at the bottom of the figure. ORFs are numbered consecutively from left to right as described in **Table 2**, and are indicated by arrows pointing to the direction of transcription.

(Scholl and Merril, 2005) and its expression is necessary only for cell lysis in the presence of high concentrations of divalent cations (Dobbins et al., 2004). It is worth mentioning that protein encoded by *orf44* had a 99% identity with SP6 Gp36 protein, whose C-terminal sequence showed a slight similarity with that of cell wall lysozymes and its lysozyme activity differed from that of the typical endolysins of similar bacteriophages (Dobbins et al., 2004; Scholl et al., 2004). Moreover, ORF52 had a 75% identity with SP6 Gp46 protein, recently identified as a peptidase_M15_3 (Oliveira et al., 2013). This protein, also identified in both K1E and K1-5 phages, is suggested to be an endolysin although without biochemical evidence.

Proteins involved in structure and assembly were encoded in more than half of the UAB_Phi78 genome, from approximately *orf37* to *orf55* (**Table 2**; **Figure 3**). Terminases (Gp40 and Gp41), head portal (Gp30), internal virion (Gp35 and Gp37), tail (Gp33 and Gp34), tail fiber (Gp38), and tail spike (Gp49) proteins were detected in this region, showing a ≥91% similarity with the corresponding proteins of the SP6 bacteriophage. However, three proteins encoded in this region (ORF50, ORF51, and ORF54) showed the highest identity (>75%) with hypothetical proteins of the K1E bacteriophage (Scholl et al., 2004) but, no homology was found for protein encoded by *orf53* in any database.

UAB_Phi78 has the protein Gp49 and the hypothetical protein encoded by *orf56*, with a high identity to the counterparts proteins of SP6 (Gp 49 and Gp50 proteins; **Table 2**) which have been predicted as receptor-binding proteins able to interact with two distinct receptors in the polysaccharide. SP6 Gp49 protein must interact with the *Salmonella* O-antigen because is closely related to the P22 tail spike protein (Gp9) with endorhamnosidase activity that cleaves the α 1,3-O-glycosidic bond between the repeating tetrasaccharide units of this antigen (Iwashita and Kanegasaki, 1976; Scholl et al., 2004). The second receptor, distinct from O-antigen and recognized by Gp50, was predicted for SP6 bacteriophage because this phage infected a *galE* mutant of *S.* Typhimurium LT2 (Scholl et al., 2004; Nguyen et al., 2012). Similarly, we hypothesized that bacteriophage UAB_Phi78 would recognize two receptors. In this sense, the bacteriophage UAB_Phi78 infected *galE* mutant of *S.* Typhimurium LT2 but not deep rough (*rfa*) mutants (data not shown). It must be noted that the two other bacteriophages studied here did not infect those mutants (data not shown).

After analysis using CoreGenes (Turner et al., 2013), UAB_Phi78 and SP6 bacteriophages have ∼83% of proteins in common. The Rho-independent terminators were in the same position in both genomes, although their sequences showed <56% similarity (Dobbins et al., 2004). The main differences between the two bacteriophages occur at the beginning of the sequence of the UAB_Phi78 genome and in the region between DNA primase and DNA polymerase, where there are many genes encoding proteins without defined functions according to the NCBI databases, including a hypothetical protein with unknown function that is also present in bacteriophage K1E (Gp12). Moreover, UAB_Phi78 shared 80 and 69%, respectively

TABLE 3 | Features of bacteriophage UAB_Phi87 genome, ORFs, gene products, and functional assignments.

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best e-value
		From	To						
1		767	210	–	185		Hypothetical protein wV8_gp055 (YP_002922836.1)	97	7.E-129
2		1006	764	–	80		Hypothetical protein [*Escherichia* phage EC6] (YP_009151266.1)	88	1.E-44
3		927	1037	+	36		Hypothetical protein wV8_gp054 (YP_002922835.1)	70	7.E-04
4		1432	1250	–	60				
5		2479	2384	–	31				
6		2460	2600	+	46		Hypothetical protein wV8_gp050 (YP_002922831.1)	100	2.E-24
7		2978	2799	–	59		Hypothetical protein wV8_gp049 (YP_002922830.1)	100	6.E-36
8		3045	3302	+	85		Hypothetical protein wV8_gp048 (YP_002922829.1)	96	2.E-50
9		3693	3869	+	58				
10		3820	4035	+	71				
11	*vWFA*	5695	4280	+	471		Hypothetical protein wV8_gp047 (YP_002922828.1)	98	0
12		6147	5776	–	123		Hypothetical protein wV8_gp046 (YP_002922827.1)	98	8.E-80
13		6397	6558	+	53		Hypothetical protein Felix01p077 (YP_001504372.1)	96	5.E-29
14		6540	6773	+	77		Hypothetical protein Felix01p076 (NP_944854.1)	96	4.E-47
15		6767	7357	+	196		Hypothetical protein wV8_gp043 (YP_002922824.1)	95	3.E-123
16		7405	7776	+	123		Hypothetical protein wV8_gp042 (YP_002922823.1)	86	2.E-73
17		7773	8906	+	377		Hypothetical protein wV8_gp041 (YP_002922822.1)	93	0
18		8906	9370	+	154	Lysozyme	Lysin (lysozyme) [*Salmonella* phage FelixO1] (NP_944846.1)	99	2.E-108
19		9421	9819	+	132		Hypothetical protein Felix01p068 (NP_944844.1)	97	4.E-89
20		9812	10210	+	132		Hypothetical protein SP107_00535 [*Salmonella* phage FSL SP-107](AGF89476)	94	6.E-85
21		10210	10503	+	97		Hypothetical protein wV8_gp037 (YP_002922818.1)	97	7.E-62
22		10496	10840	+	114		Hypothetical protein wV8_gp036 (YP_002922817.1)	100	5.E-76
23		10840	11421	+	193		Hypothetical protein [*Salmonella* phage SBA-1781] (AFU63462.1)	98	4.E-138
24		11494	12039	+	181		Hypothetical protein SP107_00555 [*Salmonella* phage FSL SP-107] (YP_009219564.1)	86	5.E-112
25		12036	12254	+	72		Hypothetical protein Felix01p056 (NP_944832.1)	92	2.E-41
26		12251	12754	+	167		Hypothetical protein SP010_00552 [*Salmonella* phage FSL SP-010] (AGF88761.1)	96	7.E-116
27		12736	12831	+	31				

(Continued)

TABLE 3 | Continued

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best e-value
		From	To						
28		12831	13055	+	74		Hypothetical protein wV8_gp031 (YP_002922812.1)	99	7.E-42
29		13110	13538	+	142		Hypothetical protein wV8_gp030 (YP_002922811.1)	56	3.E-46
30		13528	13992	+	154		Hypothetical protein Felix01p051 (NP_944827.1)	91	4.E-98
31		13949	14554	+	201		Hypothetical protein Felix01p050 (NP_944826.2)	91	3.E-106
32		14632	14525	–	35				
33		15166	15017	–	49				
34		15312	15235	–	25				
35		16494	16246	–	82		Hypothetical protein Felix01p049 (NP_944825.1)	100	9.E-53
36		17090	16560	–	176		Hypothetical protein SP010_00705 [*Salmonella* phage FSL SP-010] (AGF88787.1)	91	1.E-113
37		17653	17312	–	113		Hypothetical protein Felix01p044(NP_944820.1)	88	4.E-69
38		17979	17746	–	77		Hypothetical protein wV8_gp022 (YP_002922803.1)	92	4.E-45
39		18588	18046	–	180		Hypothetical protein HB2014_24 [*Salmonella* phage HB-2014] (YP_009146269.1)	96	3.E-123
40		18877	18674	–	67		Hypothetical protein wV8_gp020 (YP_002922801.1)	95	5.E-35
41		19386	18982	+	134		Hypothetical protein wV8_gp019 (YP_002922800.1)	91	6.E-84
42		19746	19474	–	90		Hypothetical protein wV8_gp018 (YP_002922799.1)	92	6.E-55
43		20169	19837	–	110		Hypothetical protein [*Salmonella* phage SBA-1781] (AFU63421.1)	95	2.E-65
44		20459	20163	–	98		Hypothetical protein Felix01p034 (NP_944810.1)	98	5.E-63
45		21064	20552	–	170		Hypothetical protein SP010_00685 [*Salmonella* phage FSL SP-010](AGF88783.1)	96	2.E-116
46		21412	21155	–	85		Hypothetical protein wV8_gp015 (YP_002922796.1)	70	4.E-31
47		21882	21499	–	127		Hypothetical protein wV8_gp014 (YP_002922795.1)	93	9.E-82
48		21794	21901	+	35				
49		22010	22168	+	52		Hypothetical protein SP012_00635 [*Salmonella* phage FSL SP-012] (AGF88904.1)	98	3.E-27
50		22170	22325	+	51				
51		23189	22404	–	261		Phage conserved protein Felix01p025 (NP_944801.1)	98	0
52		23390	23190	–	66		Hypothetical protein wV8_gp011 (YP_002922792.1|)	98	6.E-40
53		23610	23383	–	75		Hypothetical protein Felix01p021 (NP_944797.1)	85	2.E-38

(Continued)

TABLE 3 | Continued

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best e-value
		From	To						
54		23941	23585	–	118		Hypothetical protein Felix01p019 (NP_944795.1)	96	3.E-60
55		24215	23901	–	104		Hypothetical protein Felix01p017 (NP_944793.2)	100	6.E-69
56		24481	24212	–	89		Hypothetical protein wV8_gp008 (YP_002922789.1)	99	5.E-59
57		24747	24478	–	89		Hypothetical protein Felix01p015 (NP_944791.1)	99	2.E-56
58		24988	24641	–	115		Phage conserved protein Felix01p014 (NP_944790.1)	100	3.E-78
59		25505	25041	–	154		Hypothetical protein wV8_gp005 (YP_002922786.1)	100	2.E-104
60		26211	25516	–	231	PseT polynucleotide 5′-kinase/3′-phosphatase	Putative PseT polynucleotide 5′-kinase/3′-phosphatase [*Salmonella* phage FSL SP-010] (AGF88668.1)	99	2.E-166
61		26737	26189	–	182		Hypothetical protein wV8_gp003 (YP_002922784.1)	99	2.E-128
62	*rIIB*	27947	26838	–	369		rIIB protein [*Escherichia* phage wV8] (YP_002922783.1)	98	0
63	*rIIA*	30393	28027	–	788		rIIA protein [*Salmonella* phage FSL SP-010] (AGF88671.1)	98	0
64		30598	30422	–	58		Hypothetical membrane protein Felix01p243 (NP_945023.1)	95	8.E-32
65		30915	30580	–	111		Hypothetical protein SP010_00075 [*Salmonella* phage FSL SP-010] (AGF88673.1)	97	4.E-73
66	*nadV*	32750	30969	–	593	Nicotinate phosphoribosyltransferase	Putative nicotinate phosphoribosyltransferase [*Salmonella* phage FSL SP-107](AGF89421.1)	96	0
67	*prsA*	33677	32796	–	293	Ribose-phosphate pyrophosphokinase	Putative ribose-phosphate pyrophosphokinase [*Salmonella* phage FSL SP-107] (AGF89420.1)	99	0
68		33971	33693	–	92		Hypothetical protein Felix01p233 (NP_945013.1)	96	1.E-59
69		34479	33964	–	171		Hypothetical protein SP10700240 [*Salmonella* phage FSL SP-107] (AGF89418.1)	96	8.E-121
70		34851	34531	–	106		Hypothetical protein Felix01p227 (NP_945007.1)	97	8.E-67
71		35111	34854	–	86		Hypothetical protein wV8_gp132 (YP_002922914.1)	99	6.E-52
72	*nrdG*	35721	35236	–	161	Anaerobic NTP reductase	NrdG, small subunit [*Escherichia* phage wV8] (YP_002922912.1)	95	2.E-112
73		36176	35781	–	131		Hypothetical protein SP10700210 [*Salmonella* phage FSL SP-107] (AGF89412.1)	95	5.E-89
74		36373	36173	–	66		Hypothetical membrane protein Felix01p221 (NP_945001.1)	97	2.E-37
75		36474	36349	–	41		Hypothetical membrane protein Felix01p220 (NP_945000.2)	90	3.E-17

(Continued)

TABLE 3 | Continued

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best e-value
		From	To						
76	*nrdD*	37382	36528	–	284	Anaerobic nucleoside diphosphate reductase	NrdD [*Escherichia* phage wV8] (YP_002922907.1)	99	0
77		38102	37638	–	154	Homing endonuclease	Homing endonuclease [*Salmonella* phage HB-2014] (YP_009146359.1)	96	2.E-105
78	*nrdD*	39414	38215	–	399	Anaerobic nucleoside diphosphate reductase	NrdD [*Escherichia* phage wV8] (YP_002922907.1)	99	0
79		39669	39463	–	68		Hypothetical membrane protein FelixO1p218 (NP_944998.1)	97	3.E-37
80	*grxC*	39904	39662	–	80	Glutaredoxin	Putative phage glutaredoxin [Phage FelixO1] (NP_944996.1)	95	2.E-50
81	*nrdB*	40977	39904	–	357	Ribonucleoside triphosphate reductase,	Ribonucleoside triphosphate reductase, beta chain [Phage FelixO1] (NP_944994.1)	100	0
82		41315	40974	–	113		Hypothetical protein WV8_gp121 [*Escherichia* phage wV8] (YP_002922903.1)	88	1.E-67
83	*nrdA*	43521	41287	–	744	Ribonucleoside triphosphate reductase,	Ribonucleoside triphosphate reductase, alpha chain [Phage FelixO1] (NP_944991.1)	99	0
84		43807	43568	–	79		Hypothetical protein Felix01p210 (NP_944989.1)	97	4.E-51
85		44222	43899	–	107		Hypothetical membrane protein Felix01p208 (NP_944987.2)	100	2.E-72
86		44958	44203	–	251		Hypothetical protein wV8_gp117 [*Escherichia* phage wV8] (YP_002922899.1)	98	0
87		45181	44951	–	76		Hypothetical protein wV8_gp116 [*Escherichia* phage wV8] (YP_002922898.1)	96	1.E-39
88		45700	45203	–	165		Hypothetical protein wV8_gp115 [*Escherichia* phage wV8] (YP_002922897.1)	100	2.E-117
89		46730	45690	–	346	Exodeoxyribonuclease	Putative exodeoxyribonuclease [*Salmonella* phage FSL SP-107] (AGF89399.1)	98	0
90		47650	46793	–	285		Hypothetical protein wV8_gp117 [*Escherichia* phage wV8] (YP_002922894.1)	99	0
91		47872	47723	–	49		Hypothetical protein Felix01p245 (YP_001504375.1)	100	1.E-25
92		48156	47869	–	95		Hypothetical protein Felix01p246 (YP_001504374.1)	93	5.E-57
93		50110	48125	–	661	DNA primase/helicase	Putative phage DNA primase/helicase [*Escherichia* phage wV8] (YP_002922891.1)	99	0
94		50303	50103	–	66		Hypothetical protein Felix01p187 (NP_944966.1)	100	2.E-36
95		51055	50312	+	247	Kinase	Putative deoxynucleotide monophosphate kinase [*Escherichia* phage HY02] (YP_009205000.1)	98	2.E-177

(Continued)

TABLE 3 | Continued

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best e-value
		From	To						
96		51918	51118	–	266		Hypothetical protein Felix01p181 (NP_944960.1)	97	0
97		52342	51920	–	140		Hypothetical protein Felix01p180 (NP_944959.1)	99	7.E-97
98		52569	55298	–	910	DNA polymerase	Putative DNA polymerase [*Escherichia* phage wV8] (YP_002922886.1)	99	0
99		55360	55578	+	72		Hypothetical protein JH2_060 [*Escherichia* phage JH2] (YP_009219503.1)	89	1.E-36
100		55813	55499	–	104				
101		55781	55999	+	72		Hypothetical protein SP010_00270 [*Salmonella* phage FSL SP-010] (AGF88712.1)	97	3.E-42
102		55996	56142	+	48		Hypothetical protein Felix01p244 (YP_001504373.1)	100	3.E-26
103		56165	56389	+	74		Hypothetical protein Felix01p170 (NP_944949.1)	89	7.E-43
104		56343	56606	+	87		Hypothetical protein Felix01p168 (NP_944947.1)	97	2.E-39
105		56603	56821	+	72		Hypothetical protein Felix01p166 (NP_944945.1)	99	4.E-45
106		56760	57896	+	378	DNA ligase	Putative DNA ligase [Phage FelixO1] (NP_944942.1)	98	0
107		57900	58046	+	48				
108		58114	57947	–	55				
119		58457	58840	+	127		Hypothetical protein Felix01p155 (NP_944938.1)	98	1-e-85
110		58842	59054	+	70		Hypothetical protein wV8_gp093 [*Escherichia* phage wV8] (YP_002922875.1)	97	8.E-41
111		59047	59346	+	99	Transcriptional regulatory protein	Putative transcriptional regulatory protein wV8_gp092 (YP_002922874.1)	98	1.E-65
112		59348	59707	+	119		Hypothetical protein wV8_gp091 (YP_002922873.1)	99	4.E-80
113		59721	60236	+	171		Hypothetical protein wV8_gp090 (YP_002922872.1)	98	9.E-41
114		60237	60497	+	86		Hypothetical protein wV8_gp089 (YP_002922871.1)	92	3.E-52
115	*frd*	60494	61039	+	181	Dihydrofolate reductase	Dihydrofolate reductase [*Escherichia* phage wV8] (YP_002922870.1)	94	8e–122
116	*td*	61041	61940	+	299	Thymidylate synthase	Thymidylate synthase [*Salmonella* phage FSL SP-107] (AGF89371.1)	99	0
117		62347	61976	-	123		Hypothetical protein wV8_gp086 (YP_002922868.1)	98	4.E-80
118		62541	62344	–	65		Hypothetical protein wV8_gp085 (YP_002922867.1)	100	9.E-36
119		64968	62620	–	782	Tail fiber protein	Putative tail fiber protein [Phage FelixO1] NP_944923.1)	77	0

(Continued)

TABLE 3 | Continued

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best e-value
		From	To						
120		66185	65019	–	388	Tail fiber protein	Putative tail fiber protein GP37 [Phage FelixO1] (NP_944921.1)	96	0
121		66490	66188	–	100		Hypothetical protein Felix01p141 (NP_944920.1)	99	2.E-65
122		67347	66490	–	285		Hypothetical protein Felix01p139 (NP_944918.1)	99	0
123		68819	67350	–	489	Baseplate protein	Putative baseplate component [*Salmonella* phage FSL SP-107] (AGF89443.1)	98	0
124		69238	68819	–	139		Phage conserved protein [Phage FelixO1] (NP_944914.1)	100	8.E-97
125		69861	69238	–	207	Baseplate protein	Putative baseplate protein [Phage FelixO1] (NP_944912.1)	99	1.E-152
126		70838	69861	–	325		Hypothetical protein wV8_gp077 (YP_002922859.1)	99	0
127		71179	70838	–	113		Hypothetical protein wV8_gp076 (YP_002922858.1)	100	5.E-77
128		71979	71179	–	266		Hypothetical protein wV8_gp075 (YP_002922857.1)	97	0
129		74210	71979	–	743	Tape measure domain	Hypothetical protein wV8_gp074 (YP_002922856.1)	99	0
130		74449	74210	–	79		Hypothetical protein Felix01p121 (NP_944900.1)	100	2.E-41
131		74850	74452	–	132		Hypothetical protein Felix01p120 (NP_944899.1)	100	6.E-89
132		75370	74924	–	148		Hypothetical protein wV8_gp071 (YP_002922853.1)	100	5.E104
133		76738	75386	–	450		Phage conserved structural protein [Phage FelixO1] (NP_944896.1)	97	0
134		77338	76739	–	199		Hypothetical protein Felix01p116 (NP_944895.1)	100	3.E-142
135		77714	77313	–	133		Hypothetical protein wV8_gp068 (YP_002922850.1)	99	1.E-92
136		78193	77711	–	160		Phage conserved protein [Salmonella phage FelixO1] (NP_944893.1)	99	5.E-111
137		78642	78193	–	149		Hypothetical protein Felix01p113 (NP_944892.1)	100	2.E-104
138		79767	78664	–	367	Major capsid protein	Major capsid protein [Phage FelixO1] (NP_944891.1)	99	0
139		80178	79801	–	125		Hypothetical protein Felix01p111 (NP_944890.1)	94	1.E-79
140		81536	80190	–	448	Protease	Putative head maturation protease [Phage FelixO1] (NP_944888.1)	99	0
141		81880	81548	–	110		Hypothetical protein Felix01p108 (NP_944887.1)	99	9.E-72
142		82380	81880	–	166		Hypothetical protein HB2014_56 [Salmonella phage HB-2014] (YP_009146299.1)	99	4.E-115
143		83846	82380	–	488		Hypothetical protein wV8_gp059 (YP_002922841.1)	99	0
144		85464	83863	–	533	Terminase	Terminase, large subunit [Phage FelixO1] (NP_944884.1)	100	0

(Continued)

TABLE 3 | Continued

ORF	Gene	Position (nt)		Strand	No of amino acids	Predictive function	Closest hit (Accession number)	% Amino acid identity	Best e-value
		From	To						
145		85686	85486	–	66		Hypothetical protein wV8_gp057 (YP_002922839.1)	100	4.E-37
146		85887	85711	–	58				
147		85971	85879	–	31		Hypothetical protein Felix01p101 (NP_944880.1)	96	4.E-18
148		86819	86085	–	244		Hypothetical protein Felix01p100 (NP_944879.1)	97	4.E-166

Gene numbers correspond with their predicted function, if known, followed by the nature of the evidence that supports the functional classification. Genes with no functional prediction, but with significant sequence similarity to genes in the NCBI database as determined by BLASTP are also listed.

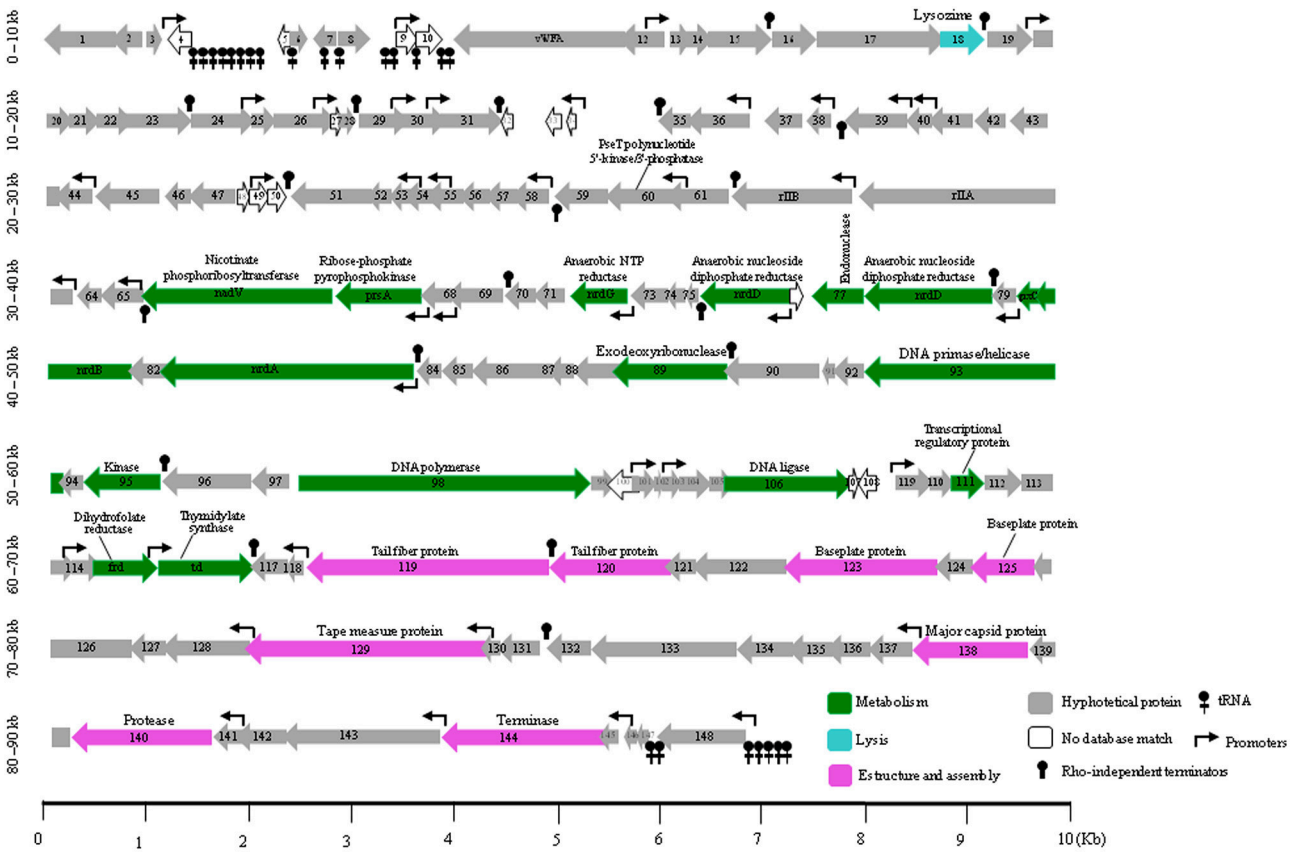

FIGURE 4 | Genomic structure of UAB_Phi87, including the Rho-independent terminators and tRNAs. Arrows represent genes, and the different colors identify the functional category into which the homologous genes were classified. Gene functions are indicated where they are known. The color code for gene function is provided at the bottom of the figure. ORFs are numbered consecutively from left to right as described in **Table 3**, and are indicated by arrows pointing to the direction of transcription.

of its proteome with those of *E. coli* bacteriophages as K1-5 and K1E.

Therefore, UAB_Phi78 belongs to *Sp6likevirus* genus of the *Podoviridae* family (Lavigne et al., 2008), which includes >35% of *Salmonella* bacteriophages (Abedon et al., 2011). An alignment of the annotated genomes of these four bacteriophages using Mauve reveals that their shared genes are largely collinear, with few noticeable differences at ∼1–2, 6.7, 22, and 39 Kb on the UAB_Phi78 genome with respect to the others (**Figure 2**).

Genome Analysis of UAB_Phi87

The complete sequenced genome of UAB_Phi87 consisted of 87,669 bp, with DTR of 608 bp and with a G+C percentage of 38.9%, clearly lower than that of *Salmonella* (52.2%). The

UAB_Phi87 genome contained 210 putative ORFs, of which 148 were finally selected (**Table 3**); the remaining 62 were in regions that overlap with these 148 ORFs. Putative functions could be assigned only to 29 (19%) of the 148 ORFs based on protein sequence similarities. The other 119 ORFs consisted of hypothetical proteins without assigned function. Of these, 104 showed high similarities with hypothetical proteins of bacteriophages Felix O1 of *Salmonella*, wV8 of *E. coli*, and in a lesser extent of *Salmonella* FSL SP107, FSL SP010, and FSL SP012. Fifteen of these 119 ORFs were apparently unique to UAB_Phi87 and they lacked similarity with sequences deposited in the databases. Potential Shine-Dalgarno sequences were highly conserved (AGGAGGA) and, the mean distance between this consensus sequence and the majority of RBS was 14 bp. Up to 42 hypothetical promoters, with highly conserved consensus sequences at −10 (TATAAT) and −35 (TTGACA), were detected (Table S1). The high degree of conservation of these sequences and their similarity with those of prokaryote promoters could be a general advantage for phage, as following infection they would be recognized by host bacteria. Twenty Rho-independent terminators were identified by FindTerm (**Figure 4**). Almost all of the ORFs (146 out of 148) started with an ATG codon; the exceptions were *orf118* and *orf144*, in which TTG was the start signal. As for the stop codons, most ORFs contained a TAA codon (67.1%). TGA was present in 25.5% of the remaining ORFs and TAG in 7.4%. The genome of UAB_Phi87 contains 23 tRNA genes and three of them (13, 18, and 20) may code pseudo-tRNA (Table S2). From the 20 functional tRNAs, 9 were found to be present at a frequency 1.5 times higher in the phage than in *Salmonella*. The high number of tRNA has been also documented in other *Felixounalikeviruses* (Whichard et al., 2010). Their presence seems to compensate for differences in codon usage between the phage and the host and to enable a positive impact on translation of phage-derived mRNA and its infectivity (Bailly-Bechet et al., 2007). Moreover, and similar to bacteriophage Felix O1, the presence of a Met tRNA suggested a positive role for this tRNA in translational initiation in phage-infected cells (Whichard et al., 2010).

The UAB_Phi87 ORFs encoding proteins with known functions were classified into three functional groups (**Figure 4**). The first one included proteins involved in nucleotide metabolism, which would allow phage replication and transcriptional control of the host machinery. Thus, DNA polymerase (*orf98*), DNA primase/helicase (*orf93*), DNA ligase (*orf106*), and other proteins encoded by genes *frd, nadV, nrdA, nrdB, nrdD, nrdG, prsA,* and *td* involved in the nucleotide metabolism were identified and presented an identity ≥94% with the counterpart proteins of the bacteriophages Felix O1 and FSL SP107 of *Salmonella*, and wV8 of *E. coli.* As it has been reported for some bacteriophages of *Felixounalikeviruses* genus (Moreno Switt et al., 2013), one split gene (*nrdD)* encoding the anaerobic ribonucleotide reductase was identified in UAB_Phi87. A gene (*orf77*) encoding a putative homing endonuclease interrupted the *nrdD* gene. In addition, this genetic structure also was in HB-2014 and JH2 phages of the *Myoviridae* family as we determined by bioinformatic analysis. The UAB_Phi87 homing endonuclease had an identity of 95–96% with the counterpart protein of all these bacteriophages and a 53% to that of the JSE bacteriophage, which belongs to the T4 group of bacteriophages infecting *E. coli*. Bacteriophages of this group, as T4 and JSE, and those of the *Felixounalikeviruses* genus typically possess several homing endonucleases (Whichard et al., 2010). Thus, T4 and Felix O1 bacteriophages encodes for 15 and 6 homing endonucleases, respectively. In contrast, in the UAB_Phi87 genome only a gene coding a homing endonuclease was identified.

The unique gene (*orf18*) with a clear function in lysis encoded a lysin with a 99% identity with the counterpart of Felix O1 bacteriophage. As in this phage, UAB_Phi87 lacks a holin gene adjacent to the lysin gene. Thus, as suggested for Felix O1 (Whichard et al., 2010), one as yet unidentified protein with unknown function located elsewhere in the UAB_Phi87 genome may assume that function. The UAB_Phi87 genome also contains *rIIA* and *rIIB* genes, first described in bacteriophage T4 (Miller et al., 2003), which encoded membrane-associated proteins of poorly understood function in this phage. It has been suggested that both could be indirectly involved in lysis inhibition, perhaps by perturbing membrane functions (Burch et al., 2011) when bacterial cells are reinfected by other T4 bacteriophages. It must be noted that UAB_Phi87 DNA was detected inside infected cells more than 100 min by PCR

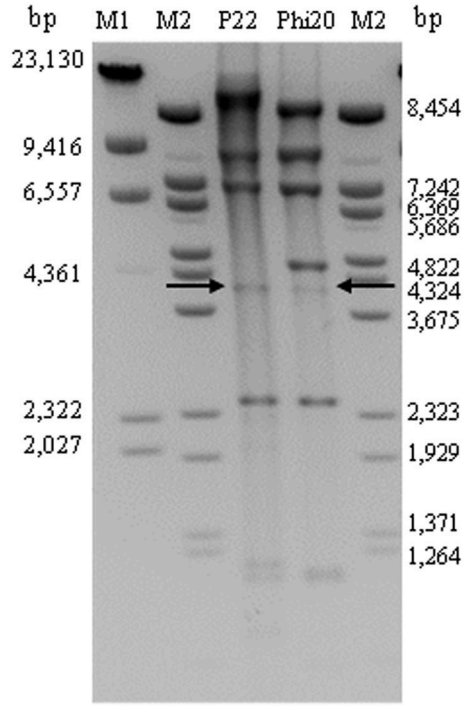

FIGURE 5 | Determination of genome ends of UAB_Phi20 phage after digestion with EcoRI enzyme. Genome of bacteriophage P22 digested with *Eco*RI was used as control. Arrows indicate the 4007-bp fragment containing the *pac* sequence. Lambda DNA digested with *Hin*dIII (M1) or *Bst*EII (M2) were used as molecular markers. Sizes (bp) are indicated on both sides of the image.

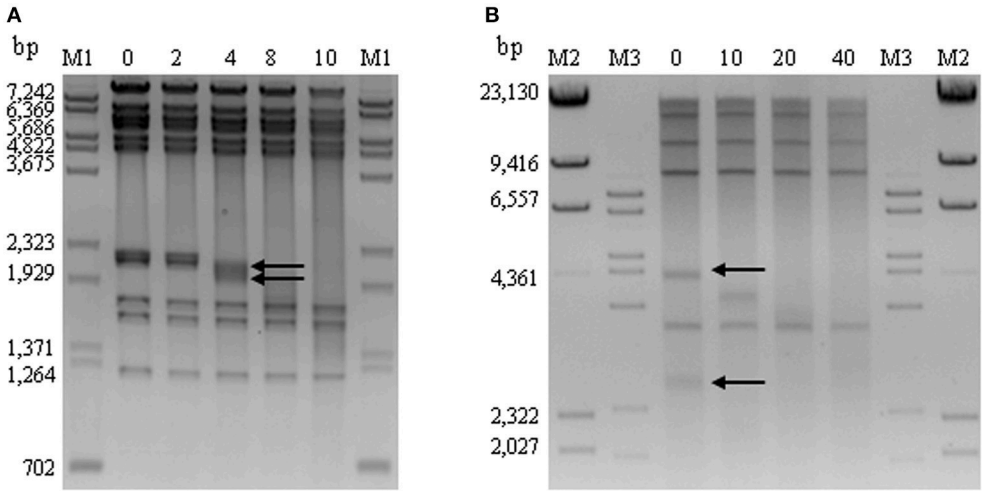

FIGURE 6 | Time-limited digestion with *Bal*31 exonuclease of UAB_Phi78 and UAB_Phi87 DNA followed by digestion with *Hin*dIII and *Spe*I, respectively. Arrows indicate the sequentially degraded DNA bands of 2200 and 2080 bp for UAB_Phi78 **(A)** and of 4322 and 2819 bp for UAB_Phi87 **(B)**. M: marker lanes containing a mixture of λ DNA digested with *Bst*EII and φX174 digested with *Hin*fI (M1), λ-DNA-digested *Hin*dIII (M2), and λ-DNA-digested *Bst*EII (M3). Sizes (bp) are indicated on the left side of the images.

amplification (data not shown) which could be related to this phenomenon.

The third functional group contained structure and assembly proteins and included tail fiber (ORF119 and ORF120), baseplate (ORF123 and ORF125), tape measure (ORF129), major capsid (ORF138) proteins, and a putative head maturation protease (ORF140). All of them presented a high identity with their counterparts of FelixO1, FSL SP-107, and wV8 phages. Only, ORF119 showed lower identity (77%) with respect to the corresponding putative tail fiber of FelixO1 (**Table 3**). At difference of many phages and similar to Felix O1 phage, only a large terminase (ORF144) was identified in the UAB_Phi87 genome (Whichard et al., 2010) with a 100% of identity. As it had been reported these large terminases presented similarity with *Erwinia amylovora* ΦEa21-4 phage, and wV8 and rV5 phages which infected *E. coli* (Whichard et al., 2010).

After CoreGenes analysis, the proteome of UAB_Phi87 shared $\leq$90% with those of FelixO1 and wV8. This allows classifying UAB_Phi87 as belonging to *Felixounalikevirus* genus of *Myoviridae* family. A MAUVE comparison of these four genomes agrees with the results of protein-by-protein comparison, and revealed the mosaic structure of the UAB_Phi87 genome and also its high similarity in terms of both genetic content and functional organization with the genomes of the other bacteriophages (**Figure 2**).

Determination of the Genome Ends of UAB_Phi20, UAB_Phi78, and UAB_Phi87 Bacteriophages

Six types of ends are well-known in the lineal dsDNA contained in the tailed-bacteriophage virions: (i) single-stranded cohesive ends (*cos* ends), (ii) circularly permuted DTR, (iii) short, several hundred base pairs exact DTR, (iv) long, several thousand base pairs exact DTR, (v) terminal host sequences, and (vi) covalently bound terminal proteins (Casjens and Gilcrease, 2009). The first five types of ends are produced by the cleavage of DNA concatemers consequence of the phage DNA replication. These cleavages are closely tied with the phage DNA packaging due to terminases encoded by the phage itself.

After sequencing the genomes, we did not obtain a clear evidence of the ends of the chromosomes of UAB_Phi20, UAB_Phi78, and UAB_Phi87 bacteriophages. In order to clarify this and their packaging strategy, firstly, the DNA of the phages was obtained and digested with *Eco*RV enzyme. Afterwards the restriction product was heat treated prior electrophoresis. Results did not evidence any change of the restriction patterns of DNA treated and untreated with heat (data not shown), showing that the chromosome of these bacteriophages did not present *cos* ends.

Because of the high similarity of UAB_Phi20 genome with those of bacteriophages of the *P22likevirus* genus and the identification of a *pac* site in its genome, we believe that this phage would have circularly permuted DTR. This was confirmed by observing the under-representation of one 4007 bp DNA fragment (**Figure 5**), which would contain the *pac* sequence, in *Eco*RI digested genome. This result is expected for bacteriophages, as P22 phage, which presents this type of ends in their chromosomes (Casjens and Gilcrease, 2009) (**Figure 5**). Following, we studied if the chromosome ends of UAB_Phi78 and UAB_Phi87 bacteriophages presented DTR in the ends of their chromosomes. Time-limited treatment of their DNA with exonuclease *Bal*31 followed by digestion with *Hin*dIII and *Spe*I enzymes, respectively, revealed the disappearance of two fragments in their respective restriction

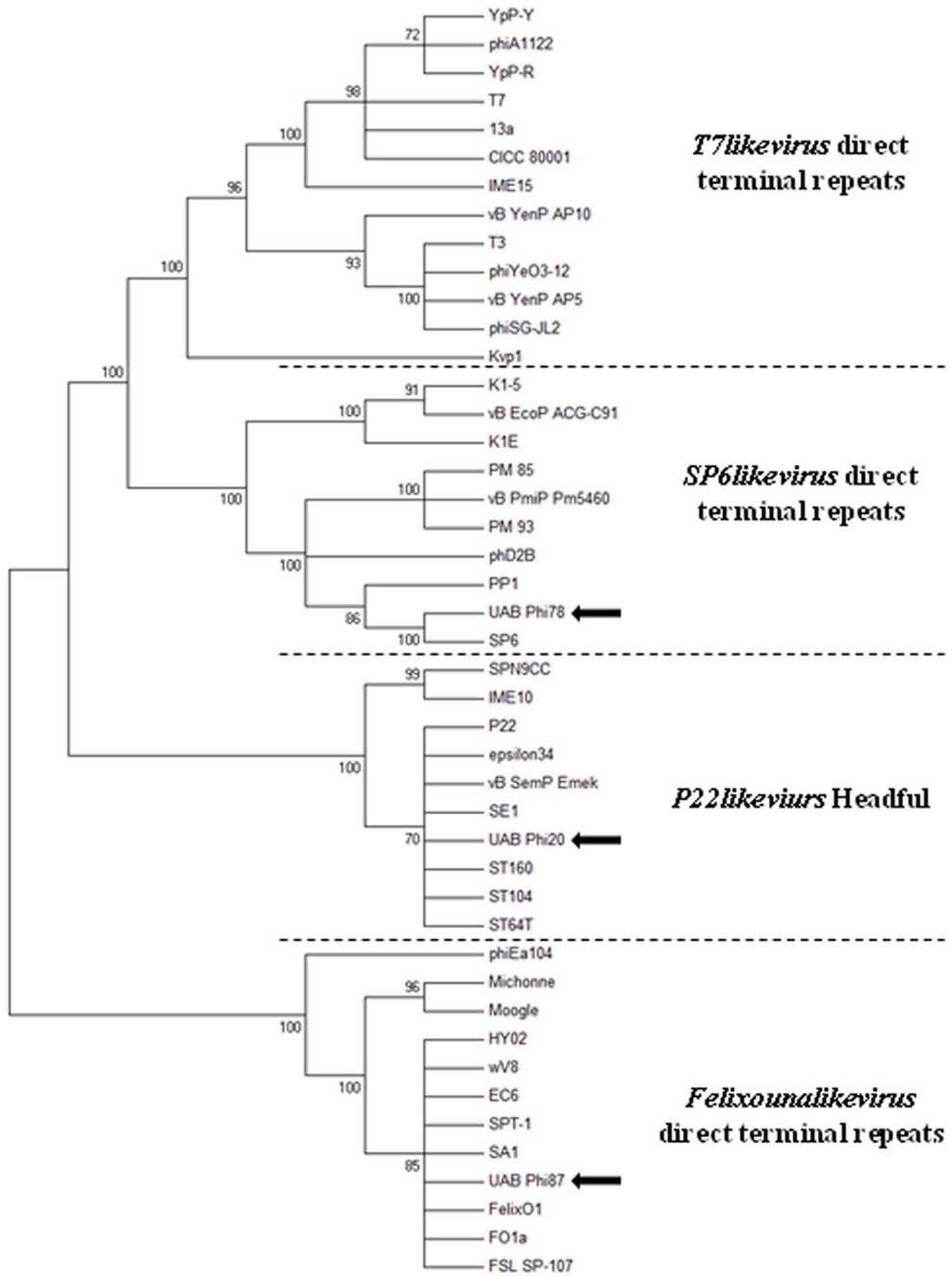

FIGURE 7 | Neighbor-joining phylogenetic tree of large terminase subunit sequences of bacteriophages UAB_Phi20, UAB_Phi78, and UAB_Phi87 (indicated by arrows) and comparison to other phages with known packaging mechanisms. Bootstrap analysis was performed with 1000 repetitions. The node of phylogenetic tree shows the bootstrap confidence values above 70%.

patterns. Thus, in UAB_Phi78, two fragments of 2212 and 2109 bp were simultaneously degraded whereas in UAB_Phi87 the disappearance of two fragments of 4322 and 2819 bp was observed (**Figure 6**). These data indicated that the degraded fragments contained the chromosomal ends of both UAB_Phi78 and UAB_Phi87 bacteriophages. According to this, specific primers were designed and used for sequencing the recovered and purified restriction fragments as templates. The primers that displayed drop-offs of the sequencing signal were selected and used to confirm the genome end sequences. For this, the respective phage genome was used as template and typical sudden drop-offs of the sequencing signal were observed (Figure S1). The analysis of the sequences obtained allowed us to identify short DTR of 179 and 608 bp for UAB_Phi78 and UAB_Phi87,

respectively (Figure S1). The size of the UAB_Phi78 DTR was similar to that described in bacteriophage SP6 (Dobbins et al., 2004; Scholl et al., 2004). Likewise, DTR of FelixO1 (Whichard et al., 2010) and FO1a (Marti, 2013) bacteriophages had a similar size to those of UAB_Phi87.

It has been reported that the packaging mechanisms, and in consequence, the type of chromosome ends of bacteriophages can be predicted comparing the amino acid sequences of the known large terminase subunits with similar enzymatic end-generating functions which usually cluster together (Casjens et al., 2005). According to this, when the neighbor-joining tree was elaborated four clusters were seen and the terminases of the UAB_Phi20, UAB_Phi78, and UAB_Phi87 bacteriophages grouped together with those of bacteriophages with similar enzymatic end-generating functions (**Figure 7**). In this sense, UAB_Phi87 large terminase clustered into the *Felixounalikevirus* DTR group, and it was highly similar to terminases of *Salmonella* phages Felix O1 and FO1a, both with DTR in their chromosome ends (Whichard et al., 2010; Marti, 2013). In the same cluster were located terminases of phages wV8 and HY02 which infect *E. coli* and others from phages infecting *E. amylovora* or *Citrobacter*. UAB_Phi78 large terminase clustered together with that of *Salmonella* phage SP6 and *Lelliottia* phage phD2B which is a *Sp6likevirus* genus with a short DTR of 262 pb (Nowicki et al., 2014). As it was expected, the UAB_Phi20 large terminase clustered into the *P22likevirus* headfull group which included bacteriophages of *P22likevirus genus* as P22, ST64T or ST160. Thus, and as it has been pointed out (Casjens et al., 2005), the structure of virion DNA ends can be accurately predicted for phages although there is no previous experimental evidences, if their putative terminase amino acid sequence falls convincingly within one of those robust groups.

CONCLUSIONS

Phage therapy is becoming an alternative or additional strategy to actual treatments of bacterial infections that can also help to diminish the emergence of antibiotic-resistant bacteria with difficult treatment. The use of bacteriophages requires a detailed characterization of these viruses. In this study, the genomes of three virulent *Salmonella* specific bacteriophages (UAB_Phi20, UAB_Phi78, and UAB_Phi87) were characterized in depth by functional genomic tools and their chromosomal ends were also determined. Detailed genome sequence analyses provided information about the three bacteriophages studied do not encode known virulence-associated or antibiotic resistance genes. The bacteriophages UAB_Phi78 and UAB_Phi87 contain terminal direct repeats in their chromosome which were identified. The UAB_Phi20 bacteriophage has a chromosome with circularly permuted DTR and it did not give rise to stable lysogens probably due to its inability to synthesize the lytic cycle repressor. This is consistent with both the complete clearance of infected-*Salmonella* cultures and the production of typical clear plaques. Genomic data and the comparison of terminases allow us the assignment of UAB_Phi20, UAB_Phi78, and UAB_Phi87 to the *P22likeviruses genus*, *SP6likeviruses* genus, and *Felixounalikeviruses* genus, respectively. This confirms the assignation reported for these bacteriophages obtained by different methods (Grose and Casjens, 2014). All the data obtained contribute to a better understanding of the biology of these phages which is necessary for the development and the use of an efficient cocktail with commercial applications in bacteriophage therapy as it has been showed (Bardina et al., 2012; Spricigo et al., 2013; Colom et al., 2015). The success of this cocktail could be attributed to the combined characteristics of the phages as their wide host-range, the different lytic cycles, and other particularities described in this study. To our knowledge, there are some reports about the use of bacteriophages closest to those studied by us but mainly in food (e.g., Whichard et al., 2003; Zinno et al., 2014), and only few in animals (e.g., Hurley et al., 2008) although with uneven results.

AUTHOR CONTRIBUTIONS

CB and DS isolated and annotated the sequences the genomes of the three bacteriophages. JC carried out the final annotation, the characterization of the packaging process of the three bacteriophages, and the analysis of terminases. MS and JO participated in the characterization of the packaging process. PC and ML participated in the design and coordination of the study and in drafting the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00545

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frontiers in Microbiology

ORIGINAL RESEARCH
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Bacteriophage T4 Infection of Stationary Phase *E. coli*: Life after Log from a Phage Perspective

Daniel Bryan[1]†, Ayman El-Shibiny[1,2,3], Zack Hobbs[1], Jillian Porter[1] and Elizabeth M. Kutter[1]*†*

[1] Kutter Bacteriophage Lab, The Evergreen State College, Olympia, WA, USA, [2] Biomedical Sciences, University of Science and Technology, Zewail City of Science and Technology, Giza, Egypt, [3] Faculty of Environmental Agricultural Sciences, Arish University, Arish, Egypt

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Oregon State University, USA

***Correspondence:**
Daniel Bryan
bryand@evergreen.edu
Elizabeth M. Kutter
kutterb@evergreen.edu

† These authors have contributed equally to this work.

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Virtually all studies of phage infections investigate bacteria growing exponentially in rich media. In nature, however, phages largely encounter non-growing cells. Bacteria entering stationary phase often activate well-studied stress defense mechanisms that drastically alter the cell, facilitating its long-term survival. An understanding of phage-host interactions in such conditions is of major importance from both an ecological and therapeutic standpoint. Here, we show that bacteriophage T4 can efficiently bind to, infect and kill *E. coli* in stationary phase, both in the presence and absence of a functional stationary-phase sigma factor, and explore the response of T4-infected stationary phase cells to the addition of fresh nutrients 5 or 24 h after that infection. An unexpected new mode of response has been identified. "Hibernation" mode is a persistent but reversible dormant state in which the infected cells make at least some phage enzymes, but halt phage development until appropriate nutrients become available before producing phage particles. Our evidence indicates that the block in hibernation mode occurs after the middle-mode stage of phage development; host DNA breakdown and the incorporation of the released nucleotides into phage DNA indicate that the enzymes of the nucleotide synthesizing complex, under middle-mode control, have been made and assembled into a functional state. Once fresh glucose and amino acids become available, the standard lytic infection process rapidly resumes and concentrations of up to 10^{11} progeny phage (an average of about 40 phage per initially present cell) are produced. All evidence is consistent with the hibernation-mode control point lying between middle mode and late mode T4 gene expression. We have also observed a "scavenger" response, where the infecting phage takes advantage of whatever few nutrients are available to produce small quantities of progeny within 2 to 5 h after infection. The scavenger response seems able to produce no more than an average of one phage per originally available cell, and few if any further progeny are produced by cells in this mode even if fresh nutrients are made available later.

Keywords: bacteriophage T4, *E. coli*, hibernation mode, multiplicity of infection (MOI), scavenger response, stationary phase, sigma S, T4 nucleotide synthesizing complex

INTRODUCTION

Bacteriophage infection has traditionally been studied using bacterial hosts growing exponentially, with active aeration, in one of a few well-studied media. The resurgence of interest in therapeutic, prophylactic, and agricultural phage applications, as well as growing awareness of the substantial environmental impact of phages, make it increasingly important to study the details of phage-host interactions under conditions that are more similar to those encountered in natural environments. Such exploration is especially important since the emergence of multi-drug resistant pathogenic bacteria has become a major public health concern (World Health Organization [WHO], 2014). Understanding how cellular changes affect phage infection under natural conditions is essential for the success of the many proposed antimicrobial phage applications. We here explore the ability of coliphage T4 to infect hosts that are in stationary phase. Such studies are particularly relevant as close relatives of this carefully studied model organism are ubiquitous in nature and have been very widely used in cocktails for treating enteric infections (Brüssow, 2005; Sarker et al., 2012; Kutter et al., 2014; Sarker et al., 2016).

T4 infection of *E. coli* has been one of the most thoroughly studied model systems in molecular biology and microbiology for over 60 years (Mathews et al., 1983; Karam et al., 1994; Miller et al., 2003). T4 infection of exponentially growing *E. coli* quickly disrupts host genome structure and expression, largely by making use of T4's complete substitution of HMdC for dC in its DNA (Kutter et al., 1994c). Transcription of all cytosine-containing DNA is blocked, translation of residual host RNA is universally cut off, host DNA is gradually degraded, and the infected cell cannot respond to a change in available carbon sources. However, it was widely believed that T4 does not multiply in stationary phase cells, since T4 plaques don't continue to grow in size after the lawn is fully formed (Benzer, 1952; Adams, 1959). Schrader et al. (1997a,b) reported that T4 was incapable of multiplying in *E. coli* AB1157 that had been starved for 24 h in Lysogeny Broth (LB), even though 94% of the T4 phage particles bound to the host. They found, in contrast, that both coliphage T7 and *P. aeruginosa* phage UT1 infected their hosts successfully in identical conditions, though with extended latent periods and reduced burst sizes; plaques of T7 on a plate will also continue to grow in size indefinitely (Yin, 1991). Other indications have been found that T4 may multiply less efficiently than T7 in the gut environment. For example, in axenic mice monocolonized with *E. coli*, the amount of T4 was seen to increase only 300-fold during transit of the gut as compared to passive transit in control axenic mice, while T7 showed a 10^6 fold increase (Weiss et al., 2009).

Optimal infection strategies might well be quite different when T4 tries to infect stationary-phase cells – as must frequently happen in the mammalian colon, *E. coli*'s primary habitat, where the microbial density is extremely high and competition for nutrients is intense. Whereas σ^{70} is the main regulator for "housekeeping" gene transcription in exponential-phase enteric bacteria, σ^S (regulated by gene RpoS) plays a central role for cell adaptation during stationary phase. There, *E. coli* maintains a basal metabolic level that lets it scavenge for nutrients from dead cells and respond quickly to a wide range of new nutrients (Hengge-Aronis, 1993; Zambrano and Kolter, 1996). Many new pathways are activated as the overall metabolic rate decreases during the transition to stationary phase, largely under the control of σ^S. *E. coli* in stationary phase have denser cell membranes, with altered phospholipids and expanded periplasmic spacing. They pack their chromosome more tightly and produce a range of protective enzymes such as catalase (Huisman et al., 1996; Págan and Mackey, 2000). The cells maintain a low level of synthesis of some proteins such as various transporters throughout stationary phase, enabling them to respond to new nutrients and to quickly resume vegetative growth (Huisman et al., 1996). The number of ribosomes is reduced (Cangelosi and Brabant, 1997), and many of those remaining are dimerized to a storage format, to be rapidly released when nutrients are supplied (Wada et al., 2000).

Here, we describe in substantial detail the patterns of the interaction when T4 infects *E. coli* 48 h after inoculation into fresh minimal medium, identifying a new "hibernation" mode of long-term interaction there, and explore the ability of T4 to produce infective centers in *E. coli* that are up to several weeks into stationary phase.

MATERIALS AND METHODS

Media

Stationary phase studies were conducted in M9 minimal medium [25 mL 20X M9 salts (120 g $Na_2HPO_4{\cdot}H_2O$, 60 g KH_2PO_4 (anhydrous), 10 g NaCl, and 20 g NH_4Cl per 790 mL DIH_2O), 0.5 mL 0.1 M $FeCl_3$, 5 mL, 0.1 M $MgSO_4$, 0.5 mL 0.1 M $CaCl_2$, per 461 mL DI H_2O, sterile filtered and supplemented with 5 mL casamino acids (CAA; 10% w/v) and 3 mL glucose (20% w/v)]. Tryptic soy broth (30 g tryptic soy broth powder per 1000 mL of DI H_2O) was used for standard cultivation of bacteria and propagation of phage. Phage buffer (10 mL 1 M Tris pH 7.6, 0.1 g gelatin, 4 g NaCl, 990 mL DI H_2O, boiled and pH balanced to 7.6 at 25°C, autoclaved) was used for phage stocks and as a diluent when titering phage.

Bacterial Strains

Studies were conducted in *E. coli* W3110 derivatives ZK126 (Δ*lacZ rpoS*+), and ZK1000 (Δ*lacZ rpoS*-) (the kind gift of Dr. Steve Finkel) and phage were routinely plated on ZK126. Amber suppressor strain CR63 from the Evergreen collection was also used. Stocks were maintained in 20% (v/v) glycerol at −80°C. All cultures were grown and infections carried out in Erlenmeyer flasks in a gyratory water bath at 37°C and 120 RPM.

Phage Strains and Their Propagation

Phage T4D was from the Evergreen collection, as was T4 gene 43 (DNA polymerase) *amber* mutant am4332. T4 was propagated in ZK126 while am4332 was propagated in amber suppressor *E. coli* strain CR63. Briefly, a 500 mL culture at ∼0.4 OD_{600nm} (OD, all

optical density readings used throughout are done at 600 nm) in TSB was infected at a multiplicity of infection (MOI) of ∼0.1, then incubated at 120 RPM and 37°C for ∼4 h before lysing with 1–2 mL of $CHCl_3$. The lysates were incubated statically at room temperature overnight to allow endogenous nucleases to degrade host DNA and then centrifuged for 10 min at 4642 × *g* to pellet bacterial debris. The supernatant was decanted away from the pellet, spun at low speed a second time, and then centrifuged for 2 h at 23,975 × *g* to obtain a concentrated phage pellet. This was resuspended without agitation in a few mL of phage buffer overnight at 4°C.

Infection of *E. coli* in Stationary Phase

Overnight cultures used for stationary phase infections or for phage plating were grown either from 3 to 5 colonies taken from plate cultures no more than 3 days old or directly from a scraping of a glycerol freezer stock stored at −80°C. This latter, less common method is used routinely in the Finkel and Kolter labs for stationary phase work, and usually gives less variable bacterial behavior in stationary phase cultures than does picking individual colonies. We adopted this technique in the fall of 2014 and the experiments presented in **Figures 1** and **2** were inoculated by this method. Stationary phase infections were performed with cultures of *E. coli* ZK126 or ZK1000 in M9 at a range of multiplicities of infection (MOI), as indicated. Either 5 or 24 h post-infection, nutrients were added to bring the concentration in the infection flask up to 0.12% w/v glucose and 0.1% w/v casamino acids. The infected culture was tracked for an additional 3–5 h after nutrient addition (representative figures showing the nutrient response of uninfected cells are provided as Supplemental Material).

Phage were tracked as plaque forming units (PFU), enumerated by taking a 30 μL sample and adding it to a microcentrifuge Eppendorf tube containing 270 μL of phage buffer and 30 μL of chloroform. The samples were shaken well and allowed to settle for at least 20 min before 30 μL of the sample was serially diluted through 96 well plates containing 270 μL of phage buffer. Then 100 μL of each appropriate dilution was added to a tube containing 3 mL of molten top agar held at 45°C and 100 μL of ZK126 at an OD of about 0.5, gently mixed well and poured onto a TSA plate. Bacterial survivors (BS) were determined by taking 30 μL samples, serial diluting through 96 well plates containing 270 μL M9 and spread plating 100 μL aseptically on round TSA plates. Optical density was determined after diluting samples either two or fourfold in M9 as needed to bring the OD below 1.0.

Duplicate samples collected at the same times had ∼100 μL of chloroform added to the dilution medium. These samples were shaken and left at least 20 min to allow the chloroform to settle; then the OD of a 600 μL aliquot was read. (Chloroform is used to indicate successful production of lysozyme, indicating some phage early gene expression. When exposed to chloroform, phage-infected cells that have already produced some lysozyme will lyse, while uninfected cells will die but generally remain structurally intact Adams, 1959).

Host DNA Degradation Experiments

To explore how stationary-phase phage infection affects the host DNA, cultures of exponential-phase ZK126 were labeled with tritiated deoxythymidine (^{3}H-dT). At OD = 0.4 on the first day, 5 mL were transferred to flasks containing 0.25 mL dA (2 mg/mL in H_2O) and 0.25 mL dT (100 μg/mL dT with 10 μCi/mL methyl ^{3}H-dT) and incubated at 37°C in a shaking water bath while the remainder was grown in parallel without label to enumerate CFUs and PFUs and check the response to nutrient addition. At 48 h, both radio-labeled and unlabeled cultures were infected with either T4D$^+$ or T4 am4332 at an MOI of about 10 phage per cell. The standard nutrient mixture was re-added to the unlabeled flasks at 5 h after infection (0.12% w/v glucose and 0.1% w/v casamino acids) to follow the ability of these infected cells to respond to nutrients by producing phage.

Labeled DNA samples were collected by spotting 50 μL samples onto filter paper disks and letting them dry for one min, placing them into a bath of 10% cold acetic acid for at least 10 min, washing them twice with cold 5% acetic acid for 10 min, then with 90% ethanol for 10 min and drying them. Samples were counted in a Packard 2200CA Tri-Carb Liquid Scintillation Counter using Perkin-Elmer Cybergold as the fluor.

[Note that *E. coli* can incorporate external deoxythymidine (dT) into its DNA only in the presence of 100 mg mL^{-1} deoxyadenosine (dA), to compete for a resident glycosidase (Boyce and Setlow, 1962)]. At least in exponential phase, T4 reuses released dT so efficiently for its own DNA synthesis that the breakdown can be observed only by using a phage mutant unable to make DNA, even when orders of magnitude more unlabeled dT is added to the culture (Kutter and Wiberg, 1968). Therefore, T4 DNA polymerase mutant am4332 was used for these studies in parallel with wild-type T4 to follow host DNA degradation in the absence of potential reincorporation of labeled host DNA into progeny phage.

RESULTS

Patterns of T4 Infection of *E. coli* in Stationary Phase:

To explore T4 infection of starved *E. coli*, a 48 h old culture of ZK126 grown in M9 supplemented with glucose and CAA was infected at a range of different MOIs. By 5–10 min post-infection, more than 90% phage binding and MOI-dependent bacterial killing was routinely observed in all conditions. The pattern of phage production is consistently different between when there are only a few infecting phage per cell and when the MOI is much higher (cf. **Figure 1A**). At lower MOIs, no phage are produced until fresh nutrients are provided, but the infected cells respond and produce phage very rapidly when glucose and CAA are re-added. This phage production is seen so quickly as to suggest that early stages of phage infection had already been initiated, rapidly yielding a substantial burst of progeny phage. We refer to this behavior as "hibernation" mode.

In contrast, with high MOIs, we also observe what we call a "scavenger" response, where some phage production begins

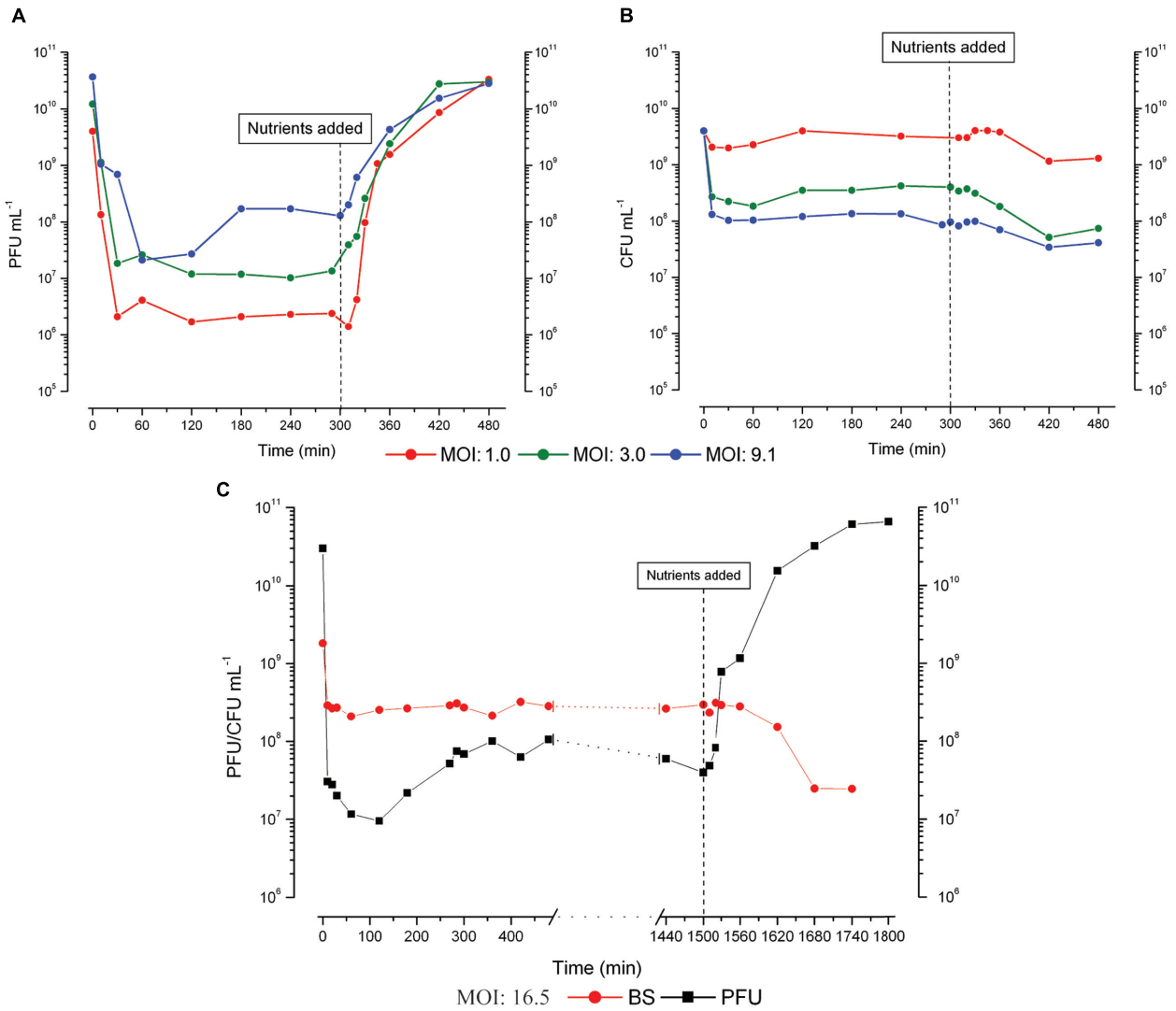

FIGURE 1 | Representative figures of (A) phage production and (B) bacterial survivors (BS) when the infection was carried out at three different multiplicity of infection (MOI) in parallel flasks split from the same 48 h old culture of *E. coli* ZK126, with 0.12% w/v glucose and 0.1% w/v CAA re-added 5 h after infection (As described in Section "Materials and Methods," chloroform is always added to the phage samples, so these values include all complete phage, both intracellular and free). (C) Bacterial killing and phage production when ZK126 was infected after 48 h with T4 at an MOI of 16.5 and no further nutrients were added until 25 h after infection. Both a long hibernation mode and a small scavenger response are observed. Supplemental Material with further representative figures showing similar behavior are provided for **Figure 1**, **Figure 2**, and **Figure 3A** as well as for the response of uninfected cells to nutrient addition.

within a couple of hours after the phage are added to the cells without any addition of fresh nutrients, seldom extending beyond 4 h after infection and showing little if any ability to produce more phage when nutrients are re-added either 5 or 24 h after phage infection, presumably due to the fact that the phage infection process has been completed.

The behavior of T4 during the infection process appears to be determined on a cell by cell basis, and at least two different sorts of responses can be observed in a single infected culture, as seen in the MOI 9 infection in **Figures 1A,B** and the MOI 16.5 infection in **Figure 1C**. There, we observed an increase in phage titer before nutrient addition, but each infected culture still rapidly produced large quantities of progeny phage after nutrient addition. Those infected stationary phase cells that enter hibernation mode are still able to respond with a similarly large burst of phage (about 200 phage per initial cell over 4 h) if the nutrients are not added until 25 h after infection (**Figure 1C**). The drop in bacterial titer starting by 2 h post nutrient addition indicates that some of the phage-producing cells have been lysing, releasing phage that can now kill any still-uninfected phage-sensitive cells.

While patterns of phage production remained quite consistent from experiment to experiment, bacterial parameters such as the titer at 48 h, extent of killing after infection at a particular MOI,

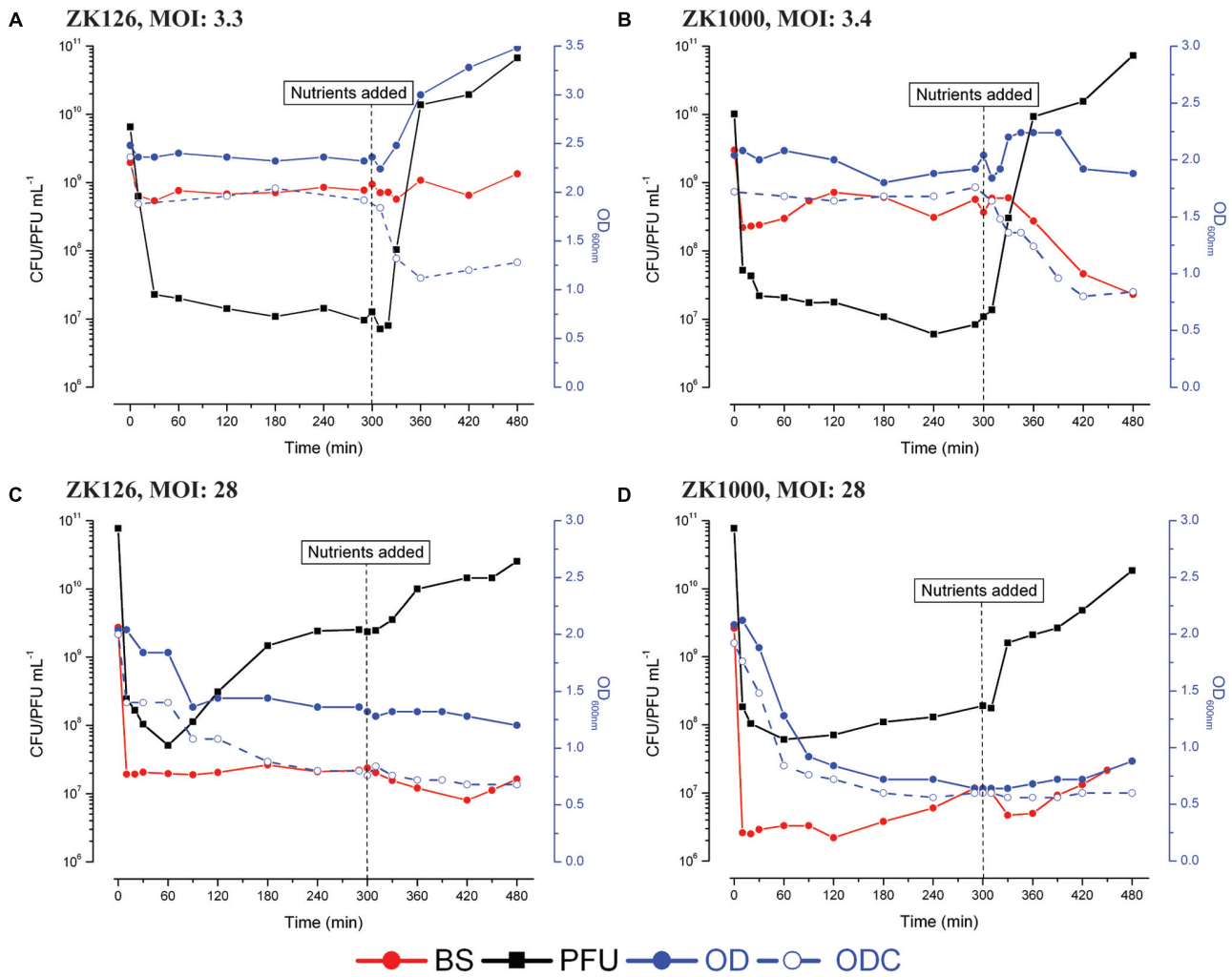

FIGURE 2 | Representative figures of phage production, bacterial killing, and optical density without (OD) and with (ODC) the addition of chloroform. 0.12% w/v glucose and 0.1% w/v CAA added 5 hr after infection. **(A)** ZK126, MOI: 3.3, **(B)** ZK1000, MOI: 3.4, **(C)** ZK126, MOI: 28, and **(D)** ZK1000, MOI: 28.

and grow-back of BS after infection varied between experiments. However, bacterial parameters in infections started in parallel from the same overnight culture remained very consistent. We hypothesize that the variation is due to differences in the initial bacterial cells in the cultures, some of which may have then developed into faster growing cells such as Growth Advantage in Stationary Phase (GASP) mutants or something that could more easily be killed etc. during the periods of stationary-phase incubation between the inoculation of the overnight culture from a freezer stock and infection of the experimental culture 72 h later (Zambrano and Kolter, 1996).

Further Characterization of Scavenger versus Hibernation Modes

Following the optical density of each culture with and without adding chloroform to the sample (ODC and OD) provides a basis for better understanding what is happening in each of the two observed modes of infection of stationary phase cells. A drop in the OD very shortly after phage addition is probably indicative of "lysis from without" due to damage caused by the infection process (Abedon, 2011). A drop in the ODC indicates that at least some lysozyme has already been produced in the cell. The T4 lysozyme gene is transcribed at low levels from an early promoter 2.9 kb away (69.9 kb on the genetic map) with an immediately adjacent strong late promoter directing much higher levels later at 67 kb on the genetic map (Kutter et al., 1994b), but the lysozyme can't reach the peptidoglycan layer as long as the inner cell membrane is intact. Exposing infected cells to chloroform induces lysis if any lysozyme has already been produced, while most uninfected cells remain unlysed even though they are killed (Adams, 1959). Thus, by following the susceptibility of the culture to lysis by chloroform, one can quickly determine whether early phage proteins are indeed being made. To explore the role of the stationary-phase sigma factor in determining response to phage, we here also include data

on infection of ZK1000, isogenic except that RpoS has been deleted.

After infection of either host at fairly low MOI, which is what would most likely occur in nature, the OD and ODC may initially drop, as does the BS, but they then are relatively stable until nutrients are added (**Figures 2A,B**). The rapid, large production of phage seen after addition of nutrients is accompanied by a substantial increase in OD, while the ODC and number of BS decrease markedly. Since the cells are not dividing, the increase in OD is probably due to gradual enlargement of each cell, as occurs in exponential-phase T4 infections (presumably to accommodate the high phage production), and phage are then being released that can kill the bacteria that survived the initial infection. (Note that the number of unadsorbed phage prior to nutrient addition is far too low to account for this killing.)

In the case of the high MOI infections (**Figures 2C,D**), where a scavenger response is more frequently observed, we saw a drop in OD in both strains fairly soon after infection. In ZK126, the OD without chloroform only fell 8% by 30 min. After 60 min gradual phage production was observed for the next 3 h, reaching an average of ∼1 phage per initial cell. By 90 min, there is an OD drop of 32%, presumably reflecting some cell lysis and release of the newly made phage. There is 46% lysis observed quite early in the presence of chloroform, implying that some lysozyme has been produced in those cells; the ODC falls an additional 16% before nutrient addition, while the OD remains relatively stable. BS titers as enumerated by CFUs remain stable until the addition of nutrients, suggesting that few free phage are present.

Shortly after infection of ZK1000, substantial lysis both with and without chloroform is observed, which may represent lysis from without and/or higher susceptibility to the early lysozyme being produced internally, something to which ZK1000, which lacks the protective adaptations of σ^S, may be more sensitive. This lysis would release some nutrients to the remaining infected and uninfected cells. Still, relatively little phage production is seen until after the addition of nutrients at 5 h (**Figure 2D**). In ZK126, most of the pre-nutrient addition OD drop seen without chloroform addition occurs much later, after phage production begins, and the majority of phage production occurs 2 to 4 h after infection. In summary, when infecting stationary phase cells T4 is capable of at least two quite different patterns of infection. T4 can appear to lie relatively dormant in the cell, in what we call "hibernation" mode. Here, it initiates the infection process but produces no progeny phage until appropriate fresh nutrients are added, whereupon it rapidly makes large numbers of phage; relatively few differences are seen whether the nutrients are added at 5 or 25 h after the initial infection (**Figures 1A–C**). Levels up to almost 10^{11} phage mL^{-1} are seen for hibernation mode. This raises questions as to at what stage of phage development this pause takes place, what triggers the pause, and what the state of the bacterial cell is during the prolonged hibernation mode of T4 infection that allows it to remain functional and able to respond to nutrients with massive production of phage.

Alternatively, infected cells may display a "scavenger" response, where T4 uses whatever resources are available to gradually make a few progeny phage per cell over the course of 2 to 5 h after infection, but shows no response to later nutrient addition. The specific details of this scavenger response remain unclear and presumably vary depending on what is available, hence its categorization as a generalized ability to respond to the conditions it encounters, rather than as a specific mode of infection.

Host DNA Breakdown in Stationary Phase

One important consequence of T4 infection of exponential-phase cells is gradual breakdown of the host DNA. This does not happen immediately after phage infection, although host transcription is shut off very rapidly. Rather, the release of mononucleotides largely parallels the production of progeny DNA (Kutter and Wiberg, 1968), while the residual host DNA is held bound in very large chunks to the membrane by many molecules of the small T4 ndd protein. The enzymes responsible for the degradation, which work in two stages, are made following delayed-early kinetics, and the released nucleotides flow directly into the phage-encoded nucleotide synthesizing complex (Kutter et al., 1994c). Their re-utilization for phage DNA synthesis is so efficient that host DNA breakdown can only be observed by using a phage mutant defective in phage DNA synthesis.

We have now carried out similar examinations of host DNA breakdown in 2-day-old stationary-phase cells in M9 supplemented with glucose and CAA, as above. For these studies, 4 mL was taken out of the culture as soon as it reached an OD of 0.4 on the first day and ^{3}HdT as well as unlabeled dA and dT were added to this aliquot, as described in Section "Materials and Methods," to label the bacterial DNA as it is being produced. At 48 h after the start of the culture, both the radioactive and non-radioactive cultures were split in two just before phage were added: either T4D or T4 DNA polymerase mutant am4332, at an MOI of about 10 (**Figure 3**).

In our various radiolabeling experiments, including the one presented here, we saw very little phage production by T4 prior to nutrient addition. Nutrients were added at 5 h post phage addition and at that point, we saw the strong nutrient response characteristic of a predominantly hibernation mode infection, yielding over 3×10^{10} phage per ml, while no phage production was observed before or after nutrient addition with am4332 (data not shown). As seen in **Figure 3A**, after wild-type infection there is only a small drop in the amount of radioactive label present in DNA, as was also reported for T4 exponential-phase infection of *E. coli* B by Kutter and Wiberg (1968); that data is reproduced here in **Figure 3B**. After infection of the same stationary-phase cells with T4 am4332, however, there was a 40% drop in acid-precipitable ^{3}HdT by 60 min after infection, and by 120 min over half of the host DNA was in acid-soluble form. As seen, though degradation is substantially slower in stationary phase, the general patterns for both the wild-type T4 and the mutant phage unable to make DNA are quite similar to those of the 1968 work infecting exponentially growing *E. coli* B.

In the 1968 exponential-phase experiments, sucrose gradient analysis showed that the residual acid-insoluble DNA is being gradually cut into large pieces (reproduced here as **Figure 3C**) by

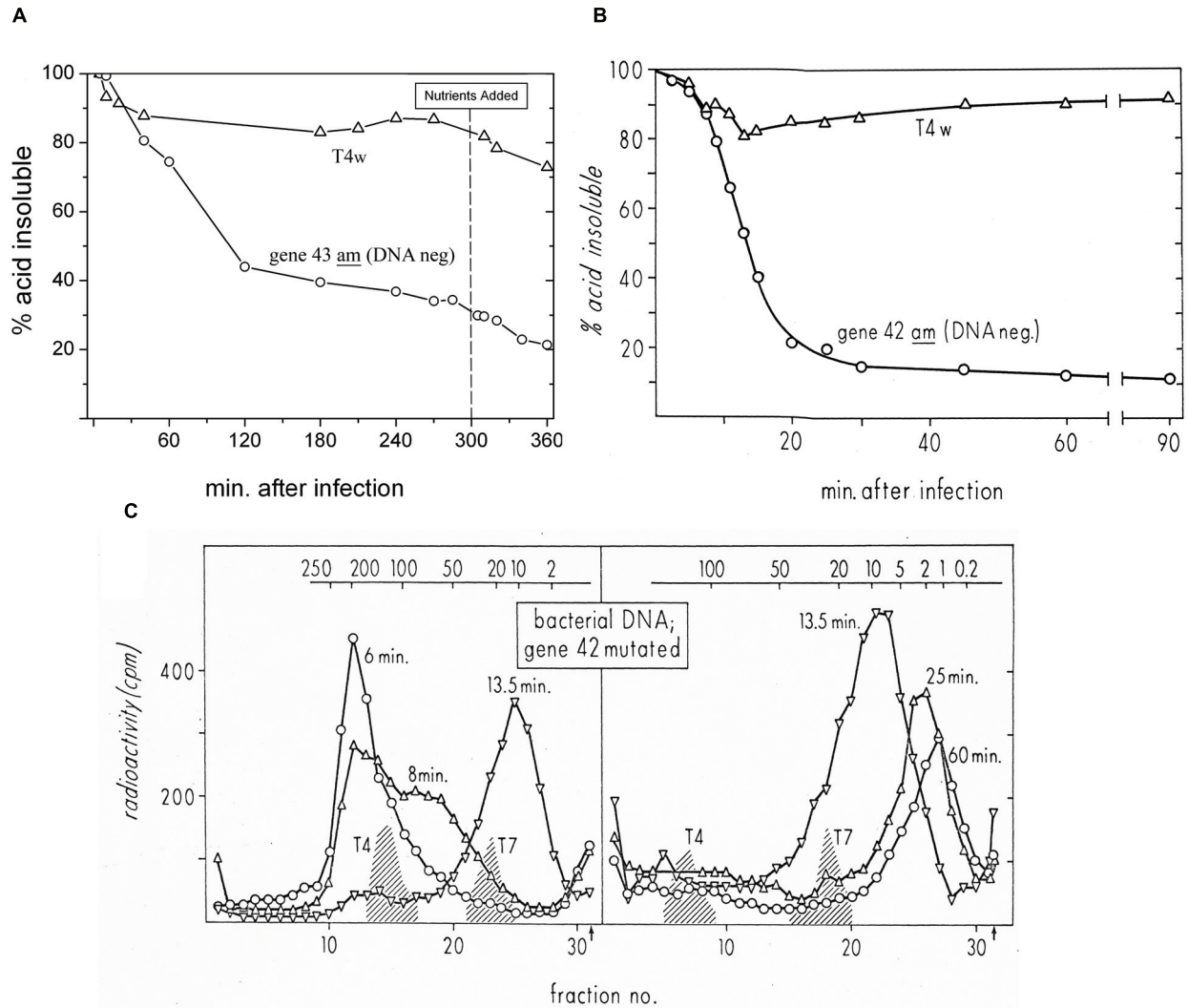

FIGURE 3 | (A) Host DNA degradation analysis of ZK126 that was labeled with tritiated thymidine during exponential growth and then infected at 48 h in stationary phase with either T4D or T4 am4332 (DNA polymerase$^-$), in parallel with infection of cultures being tracked in terms of phage production and BS. **(B)** Status of DNA labeled with tritiated thymidine over the course of similar infections of *E. coli* B with T4D and T4 amN55x5 (dCMP HMase$^-$) in exponential phase, as reported by Kutter and Wiberg (1968). Both T4 am4332 and T4 amN55x5 produce DNA$^-$ phenotype phage when grown on a non-amber suppressor strain such as *E. coli* B or ZK126. **(C)** Sucrose gradient analysis determining the size of the acid-insoluble fraction of the host DNA at various times after this exponential phase infection of *E. coli* B by T4D. "T4" and "T7" refer to phage DNAs used as sedimentation markers.

a combination of cytosine-specific phage-encoded endonucleases II and IV, while the degradation from there to mononucleotides for reutilization was shown to require T4's gene 46- and 47-encoded exonuclease, which is also involved in T4 recombination and thus not specific for cytosine DNA. In the absence of expression of genes 46 and 47, the sizes of the remaining fragments at various times after infection were the same as those seen here for T4, but all of the DNA was in the respective peaks rather than only the fraction indicated in **Figure 3B** as not yet having been degraded to mononucleotides.

Since the host DNA is so substantially degraded to an acid-soluble form after infection by the DNA polymerase mutant in stationary phase, we infer that it is similarly degraded in the parallel wild-type T4 infection, as was shown in the exponential phase sucrose gradient experiments, with the released host nucleotides being efficiently re-incorporated into phage DNA. The endoII and endoIV that initiate host DNA degradation are under middle-mode regulation, as are genes 46 and 47 and the many enzymes involved in nucleotide biosynthesis and DNA replication (Cowan et al., 1994).

Additionally, since the host DNA is being degraded, it seems highly probable that the observed rapid response to added nutrients in hibernation mode is dependent on stable host proteins and/or stable host RNAs. It appears unlikely to be the result of a transcriptional response, particularly assuming that the host DNA in infected cells is broken down in two steps

in stationary phase as it is in exponential phase (**Figure 3C**), making it likely that the residual acid-insoluble DNA in cells in hibernation mode is in relatively small fragments, as it is in exponential phase. Such fragments of DNA might be large enough to be transcribed, except for the fact that one of the first-made T4 proteins, gpAlc, blocks all transcription of cytosine-containing DNA (Kutter et al., 1981). The efficiency with which the ^{3}HdTMP from host DNA degradation is reused suggests that the elaborate T4 nucleotide synthesizing complex (diagrammed in **Figure 4**) has already been produced by the time of the degradation to acid-soluble form, that it is capable of efficiently channeling the nucleotides from host DNA breakdown into the DNA replication fork as it does in exponential phase, and that enough DNA is already being synthesized to largely use those nucleotides. It thus appears most likely that the lack of phage production in hibernation mode is due to some sort of blockage of late-gene transcription and/or translation, which is quickly reversed once the glucose and CAA again become available.

Ability of *E. coli* Infected with T4 well into Stationary Phase to form Infective Centers

All of the above stationary-phase experiments were conducted at 48 h after inoculation of the bacteria into fresh medium, leaving the question of how late into stationary phase T4 can still infect *E. coli*. A simple assessment of that question was carried out by exploring the ability of T4 to form infective centers (visible as plaques) when added at very low MOI to a culture at up to 19 days after inoculation into the same medium used for the above experiments.

T4 infection of *E. coli* in exponential phase is highly efficient; when phage are added to exponential phase *E. coli* at an MOI<<1, diluted as necessary and plated, the number of infective centers observed is equal to the number of phage that were added to that sample, as determined by other methods. This is, in fact, the basis for the way phages like T4 are normally counted. To determine how well the ability to form infective centers is preserved as the bacteria progress further into stationary phase, 10^{7} phage per mL (MOI ∼0.01) were added to stationary phase *E. coli* ZK126 and ZK1000 at various times up to 19 days after inoculation. At each time, samples were taken at 15 min, 4 and 24–27 h after infection, diluted on ice through unsupplemented M9, and immediately plated using TSA plates, where ample nutrients are available (**Figure 5**). To determine the level of unadsorbed phage, a sample was also taken at 10 min after infection into phage buffer with several drops added chloroform, vortexed well, and allowed to sit for at least 20 min before further dilution in phage buffer and plating on the same host. This lyses the infected cells before they would have completed making any progeny phage, so only the unadsorbed phage can form plaques, allowing determination of the actual numbers of infected cells that are producing infective centers.

At all times tested, at least 99.9% of the phage were able to adsorb. Interestingly, even better adsorption was observed after 11 days of growth for both host strains. For at least the first 3 days, over half of the infected cells were able to make infective centers, which were stable for at least 24 h, regardless of whether or not the host had a functioning stationary-phase sigma factor. On ZK126, 10–20% of the added phages formed infective centers even after 8–11 days, and a large fraction of those were stable for over 24 h. By 19 days, only about 1% still had found hosts where they could form infective centers; those were also still stable for 24 h.

For ZK1000, where the stationary-phase sigma factor gene has been deleted, the level of binding was similar to that seen with ZK126, but only about 5% of the cells infected at 8 days after inoculation could make infective centers, and this dropped to 1% for cells infected at 11 days and beyond. These results, coupled with the greater susceptibility to early lysis at higher MOIs (**Figures 2C,D**), indicate that while σ^{S} status may not be much involved in the regulation of T4's infection patterns, it does play a role in providing a more stable host for infection under longer term and more stressful conditions.

DISCUSSION

T4 infection of exponentially growing *E. coli* quickly disrupts host genome structure and expression, largely by making use of T4's complete substitution of HMdC for dC in its DNA (Kutter et al., 1994c). Elongation of transcription of all cytosine-containing DNA is terminated and host DNA replication is disrupted. The host DNA is gradually degraded, very efficiently funneling the released nucleotides into T4's elaborate nucleotide synthesizing complex, as discussed above (**Figure 4**). In addition, translation of host mRNA is very rapidly and universally terminated (Wiberg and Karam, 1983); the mechanism for this has still not been determined, but very little of the *lac* mRNA transcribed before or just after T4 infection is bound to ribosomes, whereas virtually all *lac* mRNA in uninfected cells is ribosome associated (Kennell, 1970). At least eight new proteins, ranging from less than 10 kilodaltons (KD) to about 48 KD, are found bound to the ribosome following infection, but none of these proteins have yet been identified genetically. T4 infection also rapidly makes *E. coli* resistant to streptomycin because the ribosomes can no longer bind the antibiotic (Wiberg and Karam, 1983).

Little is known about the effects of these various mechanisms when T4 encounters cells which are in stationary phase and which therefore have made drastic metabolic and structural adaptations to the lack of cell growth. The data presented here indicate that there are at least two possible outcomes when T4 infects stationary phase cells.

Firstly, results presented here indicate that T4 has the option of responding to the starvation state of the cell by entering what, we call a "hibernation" mode, a reversible persistent dormant state in which T4 initiates phage protein synthesis but then halts phage development midstream. Once needed nutrients become available, phage development resumes and the standard lytic infection process continues to completion, producing large numbers of progeny phage. This sort of "hibernation" mode would protect the phage from environmental factors, giving the phage a safe haven for a prolonged period of time, and would also give it a competitive advantage against unbound phage

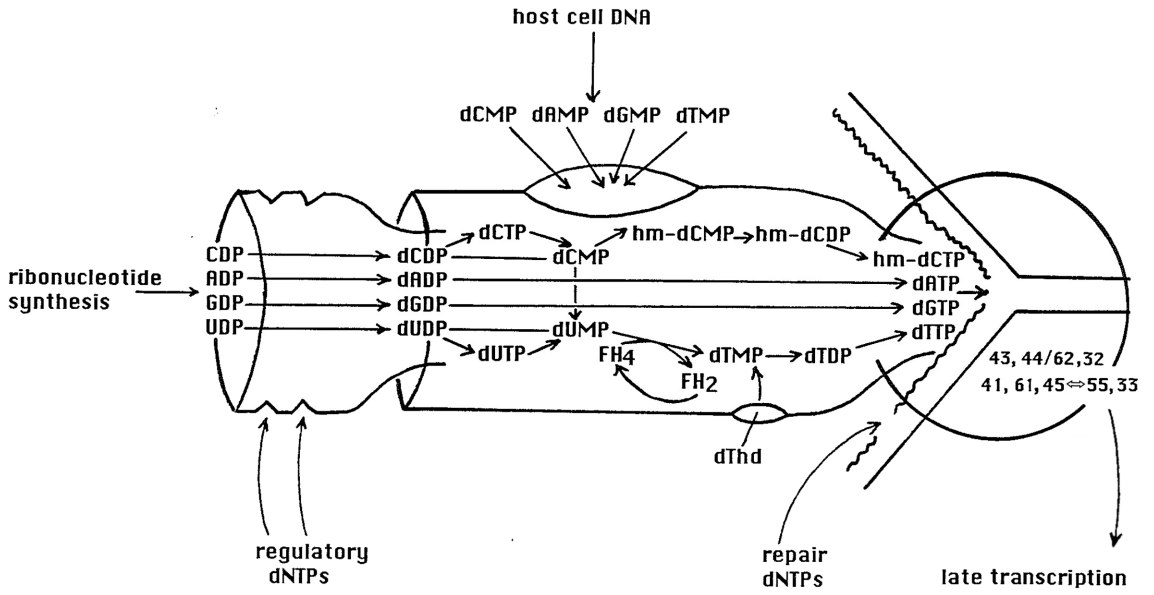

FIGURE 4 | The tight T4 nucleotide synthesizing complex, funneling NDP nucleotides very efficiently into the dNTP nucleotides needed for synthesizing T4 DNA, in precisely the 2:1 ratio of A and T to G and C required by T4. The dNMP breakdown products of host DNA are also funneled through this complex, which feeds directly into T4's DNA synthesizing complex. This unpublished diagram was designed by Chris Matthews at Oregon State University, who long led its study.

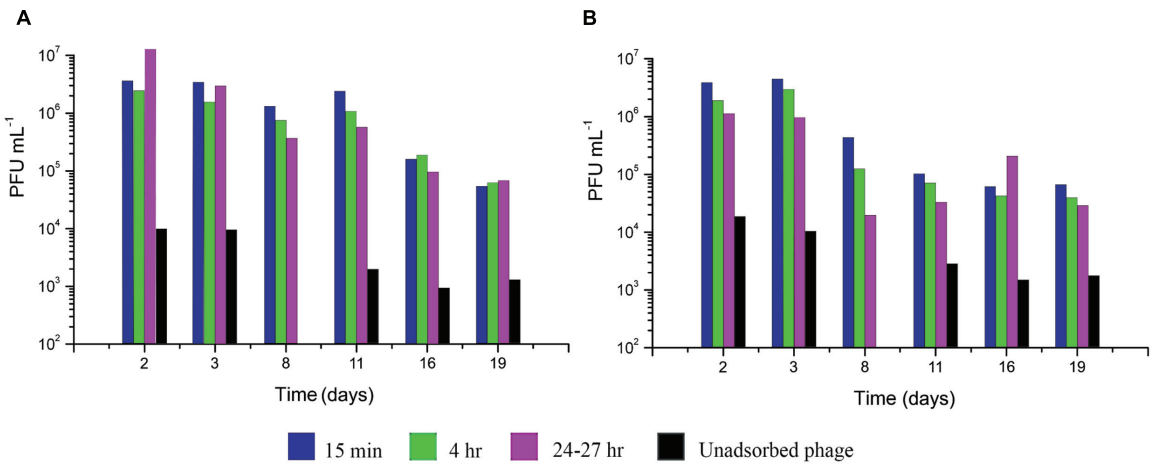

FIGURE 5 | Infective center production and stability after low MOI T4 infection at various lengths of time after *E. coli* inoculation into M9 supplemented with CAA and glucose. At each time point, 1×10^7 phage/mL were added: **(A)** in ZK126 and **(B)** in ZK1000. The number of infective centers and their stability was measured at 15 min, 4 and 24 h by serially diluting the cells and plating 0.1 ml of the appropriate dilutions on ZK126, as previously described.

when nutrient conditions abruptly change to allow bacterial growth. Interactions somewhat similar to this have sometimes been referred to as a "pseudolysogenic" state (Łoś et al., 2003; Abedon, 2009; Łoś and Wegrzyn, 2012). However, the term "pseudolysogeny" is used to describe a number of very different phenomena in the literature (Abedon, 2009; Hobbs and Abedon, 2016) so, we choose to avoid using the term here. This state is not to be considered a potential path to lysogeny, since the bacterial DNA is substantially degraded, making it is very clear that the host can never go on to replicate.

Secondly, it appears that T4 can also engage in what we call a "scavenger" response that takes advantage of whatever few nutrients are present, including nutrients released by any cells lysed from without or within, to produce small quantities of progeny. In this case, the cycle appears to run to completion within a few hours, with no ability to make further phage if nutrients later become available. This may be similar to T7's

behavior during infection of stationary phase cells, leading to its reported ability to produce a six orders of magnitude increase in phage titer while transiting the axenic mouse gut, where the level of T4 increased only 300-fold – i.e., the size of one burst in exponential phase (Weiss et al., 2009). T7's infection of stationary-phase cells on plates – allowing the plaques to grow ever larger on the plates as each infected cell releases enough phage to continue plaque enlargement even once the lawn is well into stationary phase – is probably related to that finding; a similar phenomenon is observed for some small, tailless coliphages such as phiX174.

What regulates the choice between hibernation mode and the scavenger response is unclear, but it appears to be determined on a cell-by-cell basis and not to be specifically linked to host σ^s expression. We suggest that hibernation mode is the phage production state that is actively regulated, with the scavenger response representing any possible phage production using whatever resources are available if hibernation mode is not triggered. The detection of host DNA breakdown and nucleotide reincorporation into phage DNA within the first few hours after phage infection is indicative that there is indeed middle-mode protein synthesis even before nutrient addition. However, we see no sign of an increase in completed phage particles in hibernation mode until after the addition of nutrients. All of our evidence to date suggests that it is late-mode transcription (or possibly translation) which is delayed by an unknown mechanism until adequate resources become available. We know that the infection does not progress to the production of phage and completion of the lytic cycle until after nutrient addition, but we cannot yet identify the precise point at which hibernation mode suspends phage production.

In stationary phase, we know that at least enough DNA is produced to incorporate most of the nucleotides from the host DNA. We do not yet know if progress is blocked at the level of late-gene transcription or translation or later. A very unusual sigma factor, gp55, is essential for T4 late-gene transcription (Williams et al., 1994). It recognizes a promoter that has no −35 region; it only interacts with a −10 stretch of nucleotides in the usual promoter region. However, gp55 also interacts directly through gp45 with the DNA synthesizing complex which is depicted in **Figure 4**, coupling the level of late mRNA and thus late protein synthesis to a substantial degree to the production of DNA to be packaged into the procapsids (Geiduschek and Kassavetis, 2010). During exponential phase infections, the T4 DNA presents as a long multi-branched concatemer, containing approximately 50 T4 equivalents of DNA. The DNA is packaged into the heads from the ends of the branches by a head-full mechanism, with any nicks being repaired and side branches clipped off in the process by the packaging enzymes. Since the control of late transcription is specifically linked to DNA replication at least in exponential phase, this suggests that at least some late-mode transcription may well have already occurred, and the possibility that hibernation mode control of the phage program happens at the translational level should be considered. For example, one factor which could conceivably play a role in the rapidity of the response to new nutrients is the sudden increase in functional ribosome numbers as the ribosomal dimerization characteristic of stationary-phase cells is virtually instantaneously reversed (Shimada et al., 2013).

Stationary-phase changes in the bacterial surface could also interfere with the infection process, making some phages unable either to adsorb properly or to transfer their DNA into the potential host's cytoplasm, but we see no indication of binding problems for the interaction of T4 (or coliphage T5, data not shown) with the tested lab strains of *E. coli* in stationary phase. We did, however, find that some other coliphages in our collection were far less capable of binding to stationary phase ZK126 and ZK1000 and showed no phage growth either before or after nutrient addition (data not shown).

Integration of these Observations with more Ecological Studies of T4 and Related Phages

The ability of any given phage to successfully infect stationary phase cells is likely to be influenced by its natural hosts and habitat, population reservoirs and life cycle.

Various lines of evidence indicate that T4 is specifically adapted to the colonic environment of mammals and has developed strategies to optimize survival in those conditions. For example, T4 requires specific monovalent cation concentrations most commonly found in colonic environments to efficiently bind to *E. coli*. It is incapable of binding in fresh water, and binding efficiency was greatly reduced in the high salt concentrations commonly found in sea water (Abedon, 1990). Furthermore, T4B also needs tryptophan – a nutrient not commonly found in extra-colonic environments, but, present in fecal water – to successfully bind, though T4D has no such known requirements. Furthermore, T4's optimum temperature for infection is about 37–42°C, above which phage production drops off precipitously, while the intracellular osmolarity has surprisingly little effect over a range from 58 to 630 mOsm, despite its effects on phage assembly *in vitro* (Kutter et al., 1994a).

In the ever-changing colonic environment, the ability to either produce low levels of phage as quickly as possible or to so effectively 'reserve' a host for greater future progeny production would give T4 a competitive advantage against other coliphages. A clinical study in Bangladesh showed that some of the T4-related phages orally administered to diarrheal patients experienced elevated phage titers in stool samples, suggesting that intestinal phage amplification can occur (Bourdin et al., 2013), though little or no increase was observed in Nestle's Bangladesh infant diarrheal clinical trials, where lower than expected levels of *E. coli* were found in the stools (Sarker et al., 2016). Scavenger response growth would maintain a small population of free T4 in the colonic environment. Hibernation mode, in contrast, would give T4 a longer-term survival mechanism as well as a temporal advantage when more nutrients become available, since hibernation mode infected cells start producing phage almost immediately after re-addition of usable nutrients, as opposed to having to wait until their hosts have started growing to begin the infection process. This would be consistent with the broad range of adaptive strategies known for T4 and related phages. For example, T4 has evolved a mechanism that allows infected

cells to detect related extracellular phages attempting to superinfect the cell and delay the lysis of phage-pregnant cells to release progeny for about 6 h, giving T4 an advantage where there are substantially more phage around than susceptible hosts (Paddison et al., 1998).

CONCLUSION

T4 is able to enter and take over stationary-phase cells, finding a longer-term safe haven in at least some of the cells it infects in stationary phase. In this "hibernation" mode, the phage production process pauses at some point after DNA synthesis is in progress – but before the completion of the T4 capsid – until new nutrients become available. It then produces substantial numbers of phage in short order – without having to wait through the usual lag phase before uninfected cells would start multiplying, and much faster than it would start releasing phage in exponential phase. T4 is also capable, after exposure to stationary phase cells, of producing progeny phage over a period of 2–5 h, a rate far slower than that seen in cells growing exponentially. This "scavenger" response infection renders the cell incapable of responding to fresh nutrients, and some of the cells appear to lyse very early without making progeny, especially in *E. coli* lacking the stationary-phase sigma factor. This early lysis may provide additional nutrients to any intact infected cells, allowing for a small amount of phage production without additional nutrient addition.

Further work is planned to explore what if any T4 genes are involved in hibernation mode, whether other T4-related phages share this extra capability, and how widely it has evolved within that subfamily and beyond – particularly whether it is found in the RB49/MEV12 group of phages, which do not have HMdC in their DNA and thus are more limited in their tools for taking over the host.

AUTHOR CONTRIBUTIONS

Primary responsibility for conception and design of the work: EK. Substantial contributions to the design of the work and acquisition, analysis and interpretation of the data: DB and AE-S. Substantial contribution to the acquisition, analysis and interpretation of the data: EK, ZH, and JP. Drafting the work and revising it critically for important intellectual content: EK, DB, and AE-S. Revising the work critically for important intellectual content: ZH and JP. Final approval of the version to be published AND agreement to be accountable for all aspects of the work in ensuring questions related to the accuracy or integrity of any part are appropriately investigated and resolved: EK, DB, AE-S, ZH, and JP.

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SUPPLEMENTARY MATERIAL

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The Resistance of *Vibrio cholerae* O1 El Tor Strains to the Typing Phage 919TP, a Member of K139 Phage Family

*Xiaona Shen[1,2], Jingyun Zhang[1,2], Jialiang Xu[3], Pengcheng Du[1,4], Bo Pang[1,2], Jie Li[1,2] and Biao Kan[1,2]**

[1] State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China, [2] Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou, China, [3] School of Food and Chemical Engineering, Beijing Technology and Business University, Beijing, China, [4] Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing, China

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***Correspondence:**
Biao Kan
kanbiao@icdc.cn

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Bacteriophage 919TP is a temperate phage of *Vibrio cholerae* serogroup O1 El Tor and is used as a subtyping phage in the phage-biotyping scheme in cholera surveillance in China. In this study, sequencing of the 919TP genome showed that it belonged to the *Vibrio* phage K139 family. The mechanisms conferring resistance to 919TP infection of El Tor strains were explored to help understand the subtyping basis of phage 919TP and mutations related to 919TP resistance. Among the test strains resistant to phage 919TP, most contained the temperate 919TP phage genome, which facilitated superinfection exclusion to 919TP. Our data suggested that this immunity to *Vibrio* phage 919TP occurred after absorption of the phage onto the bacteria. Other strains contained LPS receptor synthesis gene mutations that disable adsorption of phage 919TP. Several strains resistant to 919TP infection possessed unknown resistance mechanisms, since they did not contain LPS receptor mutations or temperate K139 phage genome. Further research is required to elucidate the phage infection steps involved in the resistance of these strains to phage infection.

Keywords: ***Vibrio cholerae*, bacteriophage, 919TP, K139, resistance**

INTRODUCTION

Vibrio cholerae is a Gram-negative bacterium and the causative agent of cholera. Seven pandemics of cholera have occurred worldwide over the last two centuries. Molecular subtyping and genome sequencing have revealed that different phylogenetic clones of *V. cholerae* O1 El Tor caused various epidemics during the seventh pandemic (Mutreja et al., 2011; Didelot et al., 2015). Subtyping, including biological subtyping and molecular subtyping methods, is necessary in microbiological studies and epidemiological investigations of pathogenic bacteria (Chattopadhyay et al., 1993; Chakrabarti et al., 2000). A Phage-Biotyping Scheme was developed for the subtyping of O1 El Tor strains and has been used in the cholera surveillance in China since the 1970s (Gao et al., 1984; Xiao et al., 2013). In the phage typing part of the scheme, five typing phages (named VP1 to VP5, respectively; Gao et al., 1984; Zhang et al., 2009; Li et al., 2013; Xu et al., 2013, 2014) are used and El Tor strains can be clustered into 32 distinct phage types (from 1 to 32) according to their

lytic patterns to these five phages. In the biological typing part, El Tor strains can be grouped into 12 biotypes (from a to l) according to their biological performance in lysogenicity, susceptibility to temperate phage 919TP, sorbitol fermentation, and hemolysis (Gao, 1988; Wang et al., 2009; Xiao et al., 2013). Using the phage-biotyping scheme, El Tor strains isolated from patients in epidemics could be differentiated from those isolated from environmental samples during non-epidemic periods, and the scheme has been used in the surveillance and identification of sources of cholera outbreak.

The study of phage typing mechanisms is helpful for revealing genetic differences among the different bacterial strains with the various phage types in the environment and in epidemics, for example, resistance to phage infection may confer the strain the ability to survive in the environment, with the potential of triggering an epidemic later (Faruque et al., 2005; Jensen et al., 2006). The phenotype change from sensitivity to resistance to the phage infection always results from gene mutations. In our previous studies, we found that mutations in the receptor genes (e.g., *ompW*, core oligosaccharide genes, and *O*-antigen genes) of *V. cholerae* El Tor strains may render the strains resistant to the typing phages used in the Phage-Biotyping Scheme (Zhang et al., 2009; Xu et al., 2013, 2014). Bacteria commonly avoid phage infection using mechanisms associated with phage adsorption, injection, gene replication, assembly, and release (Hyman and Abedon, 2010; Labrie et al., 2010). Additionally, integration of temperate phage into the host bacterial genome confers superinfection exclusion by a phage, as a result of which the strains carrying the temperate phage genome in their chromosome will be resistant to subsequent infection by the same phage and phages with same immunity region.

The subtyping phage 919TP is a temperate bacteriophage used in the Phage-Biotyping Scheme in cholera surveillance in China. Some *V. cholerae* O1 El Tor strains isolated during epidemic periods are sensitive to phage 919TP and lyse, while strains isolated during other epidemic periods show resistance to this phage (Wang et al., 2007; Deng et al., 2008), suggesting that these strains may belong to different genetic clones having different genetic determinants related to 919TP infection and replication. This study was initiated to identify the genetic mutation(s) in the strains resistant to 919TP infection, to reveal the genetic variance among these epidemic El Tor strains based on their phenotype (sensitivity to 919TP), and to help understand the phage-biotyping mechanism for *V. cholerae* El Tor strains. We sequenced the genome of phage 919TP and found that it belongs to the K139 phage family (Reidl and Mekalanos, 1995; Nesper et al., 1999, 2000; Kapfhammer et al., 2002). We found that 919TP-resistant *V. cholerae* strains used different strategies to avoid infection, including superinfection exclusion, receptor mutation, and as yet unknown mechanisms.

MATERIALS AND METHODS

Bacterial Strains, Phage, and Media

Phage 919TP, isolated from an overnight culture of *V. cholerae* O1 El Tor strain 919T, was propagated on the *V. cholerae* host strain SM6. A group of 116 O1 El Tor *V. cholerae* strains isolated in different years and regions in China (**Supplementary Table S1**), including 90 toxigenic strains ($ctxAB^+$) and 26 non-toxigenic strains ($ctxAB^-$), were selected for the detection of sensitivity to phage 919TP infection and identification of 919TP resistance mechanisms. El Tor strain N16961 (Heidelberg et al., 2000), whose whole genome has been sequenced and which is sensitive to phage 919TP, was also included. Unless otherwise stated, all strains were grown at 37°C in/on liquid or solid (15 g/L agar) Luria broth (LB) medium. *V. cholerae* O1 El Tor strains complemented with the phosphomannomutase (*manB*) gene were grown in media containing 100 μg/mL of ampicillin (Xu et al., 2013).

Propagation of Phage 919TP and DNA Extraction

Five milliliters of culture of strain SM6 (3×10^8 CFU/mL), mixed with phage 919TP (3×10^6 PFU) were added to 500 mL of LB medium, incubated at 37°C, and shaken for 5–7 h. The mixture was centrifuged at 8470 × *g* for 10 min to remove cell debris and then filtered through 0.22 μm pore-size filters. The filtrate was treated with DNase I (1 μg/mL) and RNase A (1 μg/mL). Solid NaCl (5.84 g/100 mL) and polyethylene glycol 8000 (10 g/100 mL) were added, and the mixture was centrifuged at 8470 × *g*. The phage particles in the precipitate were resuspended in SM buffer (Sambrook and Russell, 2001). Phage DNA was extracted as previously described (Zhang et al., 2009). DNA was precipitated with isopropanol and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) buffer solution.

Genome Sequencing of 919TP from Phage Particles and of Its Host Strain *V. cholerae* 919T

After the DNA sample was extracted, the sample quality was analyzed by gel electrophoresis. The DNA sample was then used to construct a library following shearing of the purified DNA into smaller fragments of the desired size using a Covaris S/E210 focused-ultrasonicator. The library was constructed with average insert lengths of 500 bp according to the manufacturer's instruction (The DNA Library Prep Reagent Set for Illumina, E6000S/L, NEB). The overhanging genomic sequences resulting from fragmentation were converted into blunt ends using T4 DNA polymerase, the Klenow fragment and T4 polynucleotide kinase. Following addition of an 'A' base to the 3′ end of the blunt phosphorylated DNA fragments, adapters (The NEBNext Multiplex Oligos for Illumina, E7600S, NEB) were ligated to the ends of the DNA fragments. The desired fragments were then purified following gel-electrophoresis, selectively enriched, and amplified by PCR. An index tag was introduced into the adapter at the PCR stage and a library quality test was performed. The qualified library was sequenced using the Illumina HiSeq 2000 sequencing platform. The sequence were assembled into contigs and scaffolds by using SOAPdenovo 1.04. We compared the phage 919TP genome sequence to other sequences by using Blast.

Phage Sensitivity Detection

To determine the sensitivity of *V. cholerae* strains to phage 919TP, the strains were grown for 3–5 h in LB broth, seeded in melted 0.5% agar (50°C) and poured onto LB agar plates. A 919TP phage lysate was spotted onto the seeded soft agar and plaque development was observed after overnight incubation at 37°C.

Detection of Spontaneous Production of Phage from *V. cholerae* Strains

In order to determine whether *V. cholerae* strains spontaneously produced 919TP (or K139 related phage), bacteria were grown overnight in LB broth at 37°C. The culture was collected by centrifugation at 12,000 rpm for 1 min and the supernatant was filtered (0.22-μm pore-size filters; Nalgene). The filtrate spotted onto soft agar plates seeded with *V. cholerae* O1 El Tor strain N16961 which is sensitive to 919TP infection. After that the plates cultured overnight to observe plaque formation.

In order to detect whether the supernatants of the strains which spontaneously produced phage contained phage 919TP (or K139 family related phages), the DNA was tested. Firstly, DNase was added into the supernatant and incubated at 37°C for 30 min to digest possible chromosomal DNA. Then the stopping solution (M6101; Promega) was added to the supernatant and incubated at 65°C for 10 min to stop the reaction. After that, the supernatant was incubated at 100°C for 10 min to release the phage genome. At last, the resulting supernatant was used as the PCR template for detection of four conserved *V. cholerae* genes encoding RecA protein (*recA*), thymidylate synthase (*thyA*), alpha subunit of DNA-directed RNA polymerase (*rpoA*), and subunit B of DNA gyrase (*gyrB*), and four phage 919TP genes encoding putative capsid portal protein (*orf15*), endonuclease subunit of putative terminase (*orf19*), putative integrase (*int*), and periplasmic protein (*glo*) using specific primers (**Table 1**).

Oligonucleotide Primers

To facilitate the detection of phage K139-related genome sequence in *V. cholerae* strains, primer pairs were designed to amplify eight K139 genes using PCR amplification, including *int*, *glo*, *orf15*, *orf19*, *cox* (putative regulator), *rep* (putative replication protein), *orf28* (putative endolysin), and *orf35* (putative tail fiber protein; **Table 1**). The assays were run in a 25-μL reaction mixture containing 12.5 μL of 2x *Taq* MasterMix (CW Biotech, China), 10 pmol of each forward and reverse primer, and 15–20 ng of nucleic acids under the following conditions: 94°C for 5 min; 30 cycles of 94°C for 1 min, 55–60°C for 1 min, 72°C for 1 min; and a final step of 72°C for 7 min.

Retrieval of *wbe* Gene Cluster (*O*-Antigen Biosynthesis) Data from *V. cholerae* Sequences and *wbe*-Related Gene Complementary Plasmids

The *wbe* gene cluster sequences of 11 test El Tor strains, including two sensitive strains and nine naturally resistant strains, were retrieved from our previous sequencing analyses: genomes of strains 147, 2255, 2454, 2833, 2981, X190, and 2657 were resequenced using an Illumina HiSeq 2000 on 250-bp and 6-kb paired-end libraries in 100-fold multiplexes at BGI, China (Didelot et al., 2015). The *wbe* gene clusters were assembled, and Blast searches were carried out against the reference genome of N16961. The *wbe* gene clusters of four strains (228, 1888, 2113, and 323) were obtained by sequencing of the PCR amplification products and assembly by DNASTAR (Xu et al., 2013). The *manB* deletion mutation of *V. cholerae* strain N16961 and the complementation of mutations were obtained in our laboratory (Xu et al., 2013). The complementary plasmid pBR322-rfbT, which complemented the natural *rfbT* gene mutants (serotype Inaba

TABLE 1 | Primers used in this study.

Primer	Sequence (5′–3′)
Sequencing of K139 genome	
int-mf	AGTGAGTGGCAAAGGTATG
int-mr	GGGCGTTCTGTTTCTATT
glo-mf	TTTGATAAGTGGGAGAAAG
glo-mr	ATAATAGGCGACTGAGTGA
cox-mf	CACGGGTAAGTGACAAAT
cox-mr	GTAAATGCCAAACAACGA
rep-mf	GATTGCCGCTGGCCATAAAG
rep-mr	TAAGGAGAGGCAAACGGCAG
orf15-mf	GTTTTTGCAGACGGGCTCAG
orf15-mr	CTATGCAACCGACCCGAACT
orf19-mf	AAACCGCAAAGCCTCACAAG
orf19-mr	GCTCATCAGTTACGCTCCGA
orf28-mf	AAGATGATACTCGCGGCCTG
orf28-mr	TTCTGCATCGCGCGTTTATG
orf35-mf	TTAACGCAAAAGACGCAGGC
orf35-mr	TTGCAACGTAGTGTTGCTGC
Amplification of *V. cholerae ctxB* genes	
ctxB-mf	ATTTTGAGGTGTTCCATGTG
ctxB-mr	ATAAAGCAGTCAGGTGGTCT
Amplification of *V. cholerae* specific genes	
thyA-mf	ACATGGGACGCGTGTATGG
thyA-mr	ATATGACCACCATCAGGCTTAGC
recA-mf	GTGCTGTGGATGTCATCGTTGTTG
recA-mr	CCACCACTTCTTCGCCTTCTTTGA
rpoA-mf	GAACAAATCAGCACGACACA
rpoA-mr	CACAACCTGGCATTGAAGA
gyrB-mf	ATCCATTCGCAAACTTACCAT
gyrB-mr	TTGATCGACACGCCAGA
Primers for qRT-PCR	
orf18-rt-mf	ATGGGACTTGACTCCGTTCT
orf18-rt-mr	TGCCGTTCGTTTCCTTGT
orf24-rt-mf	CGAGTCGCTTACGAACATC
orf24-rt-mr	TCTTCCTACTTTCCAATCCC
orf28-rt-mf	GTGTTCCTCGGCTTACTCA
orf28-rt-mr	TCGTTCTTTGGCGATTTT

strains), was also constructed in our laboratory (Liang et al., 2013).

Transcription Analysis of the Temperate K139 Phages in the El Tor Strains

To test if the temperate K139 phages had gene transcription activity, quantitative reverse transcription PCR (qRT-PCR) was performed to detect the transcription of the K139 genes *orf18-rt, orf 24-rt*, and *orf28-rt* (**Table 1**), which were involved in phage particle morphogenesis and cell lysis. *RecA* was used as the reference. Strain 919T was used as the positive control and strain 2657, which was sensitive to 919TP and has no K139 genome, was selected as the negative control. The *V. cholerae* strains were grown in LB medium overnight at 37°C and transferred into a new LB medium (1:100). Once the cells reached an OD_{600} of 1.0, they were collected by centrifugation at 4°C and immediately processed for RNA extraction. Total RNA was extracted using the Trizol reagent (Ambion), and chromosomal DNA was removed by treatment with a TURBO DNA-free^{TM} kit (Ambion). The purity of RNA samples were verified by UV spectrophotometry and agarose gel electrophoresis, and 1 μg of total RNA was used to synthesize the cDNA with SuperScript^{TM} III reverse transcriptase (Invitrogen), random primers (TaKaRa), dNTP mixture (TaKaRa), and RNase inhibitor (TaKaRa). qRT-PCR was performed using SYBR Green (TaKaRa) on a Bio-Rad CFX96 Real-Time PCR detection system. The assays were run in a 20-μL reaction mixture containing 10 pmol of each forward and reverse primer and 2 μL of nucleic acids under the following conditions: initial denaturation at 95°C for 3 s; 40 cycles of 95°C for 5 s; and 60°C for 30 s; followed by melting curve analysis, which revealed single amplicons with an appropriate melting temperature.

Observation of Adsorption of SYBR Gold-Stained Phage 919TP onto the Surface of Bacteria

Phage lysates with titers of at least 10^8 PFU/mL were filtered through 0.02-μm pore-size filters (6809-5002; Waterman, Germany). The filters were washed at least five times with phosphate-buffered saline (PBS) using 1 mL of PBS to extract phage. The extracted phage samples, with titers of at least 10^9 PFU/mL, were mixed with a SYBR gold nucleic acid gel stain stock solution (S11494; Invitrogen) in a 10,000:1 (vol/vol) mixture. This mixture was subsequently incubated for 20 min at 4°C for SYBR gold staining of the phage particles. Bacterial cultures, grown to an OD_{600} of 0.3, were mixed with the gold-stained phage (1:1, vol/vol), and incubated for 10 min at 37°C. Next, bacterial/phage mixtures were centrifuged at 6,000 × *g* for 3 min. The precipitate was resuspended in 600 μL of PBS, recentrifuged, and resuspended in 50 μL of fresh PBS. Phage 919TP adsorbed on the surface of bacteria was examined by confocal laser scanning microscopy (CLSM; FV500; Olympus, Japan).

RESULTS

Genome Sequence of 919TP and Comparison with the K139 Genome

Using the Illumina HiSeq 2000 sequencing platform, 100 Mb of genomic sequence data was generated from extracted 919TP phage genomic DNA. Linear DNA of 33,133 bp with a GC content of 48.92% was observed after assembly. The complete genomic sequence of phage 919TP was deposited in the GenBank database under the accession number KU504502. Following online sequence alignment[1], the 919TP genomic sequence was highly homologous to *Vibrio* phages of the K139, VPUSM8, and KAPPA families. A homologous fragment was also identified in the genome of *V. cholerae* strain MJ1236 (Grim et al., 2010). The genome sequence of phage 919TP was 99% identical to that of phage K139 with 99% query coverage. Therefore, phage 919TP was considered to belong to the K139 family.

The genome of the host *V. cholerae* strain 919T was also sequenced. A 3,955,624 bp genome with 58 scaffolds was generated from the sequence data (402 Mbp). The GC content of the genome was 47.49%. The integrated 919TP genome sequence was extracted from the genomic sequence of *V. cholerae* 919T and K139 sequence was used for Blast analysis and comparison with different K139 phages. The genomic sequence was 99% identical between different phages. A single base mutation was found in the intergenic region. A small fragment deletion, and 16 single base insertions, deletions, gene internal transitions, and transversions were found in the 10 genes. Six genes contained a change in an amino acid, including changes associated with three open reading frames (Rep/Orf18/Orf30) resulting in the production of truncated proteins. Orf22 and Orf23 gene variations in three members of the K139 phage family occurred as a result of one new gene. Orf35 contains a transformation from a non-polar hydrophobic amino acid to a polar neutral amino acid (proline to serine).

Phage strain sequences (919TP, K139, VPUSM8, and KAPPA) were also compared with the phage genome sequences integrated into the lysogenic *V. cholerae* MJ1236 and *V. cholerae* 919T genomes. The comparisons showed reverse transformations between the head and tail (**Figure 1**). The F fragment in front of the attP site in the K139 and 919TP mature phage particles predominantly exists at the head of the phage genome. However, the F fragment that resulted from integration into the two *V. cholerae* strains shifted to generate a joint genomic tail with the attP site. Indeed, the resultant F fragment replicated in a manner similar to the whole mature phage genome by initially forming a ring, and then integrating into bacterial genomic attP locus specific sites. The genomic sequences encoding the head lengths of the analyzed phages differed in length. The K139 and 919TP phage heads were similar to each other. The head and tail sequences of the kappa phage were similar to the genome sequences of the phages integrated into the lysogenic *V. cholerae* MJ1236 and *V. cholerae* 919T genomes; the head

[1] http://blast.ncbi.nlm.nih.gov/Blast.cgi

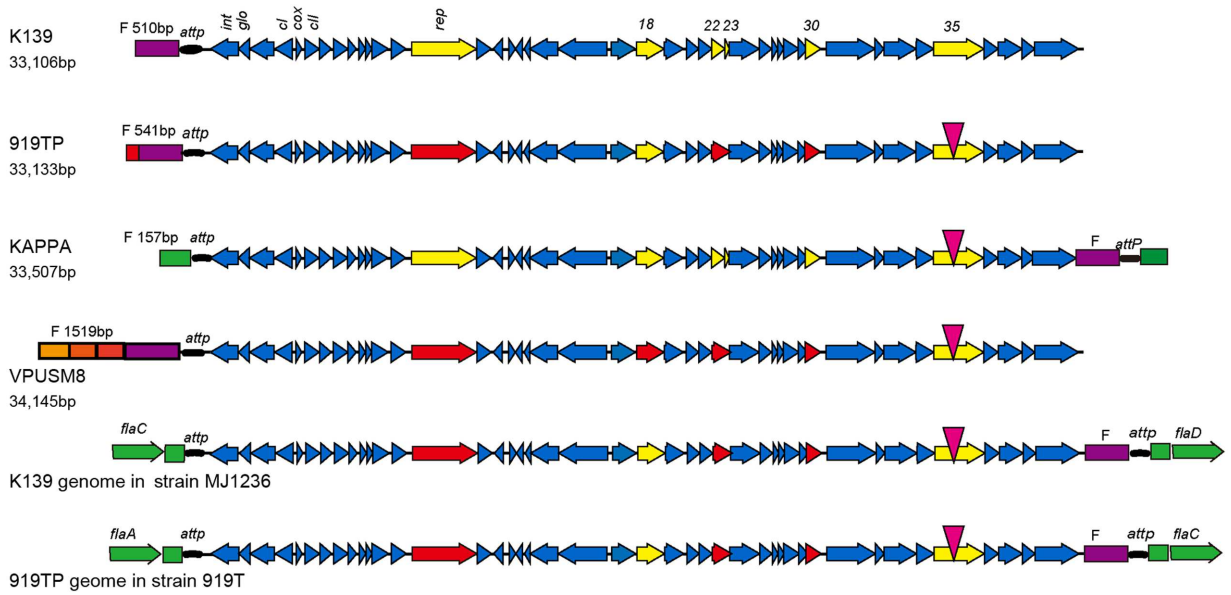

FIGURE 1 | Genome structure of phage K139 and comparison with K139-related phages. Following sequence analysis, six genes (*rep, orf18*, *orf22*, *orf23*, *orf30*, and *orf35*) contained mutations as indicated by yellow arrows. A point mutation was observed in the *orf35* gene, indicated by an inverted red triangle. The other five genes were mutated at the head or tail of the genome as indicated by red arrows. *orf22* and *orf23* mutated together to form a new hybrid gene. Sequences of the head and end of the phages are indicated by different colors. The head sequence of the phage is indicated in violet. *V. cholerae* derived sequence is indicated in green.

gene fragment came from the *V. cholerae* sequence. The head of the VPUSM8 fragment (1,519 bp) contained three short fragments and one F fragment. The three short fragments were 100% homologous to mature phage sequences in three different positions.

The K139 phage integrated in strains MJ1236 and 919T in the same site-specific integration sequence but, interestingly, the site-specific sequence was located at different positions in the bacterial genome, between *flaC* and *flaD* in MJ1236, and between *flaA* and *flaC* in 919T.

Strain Selection for the Analysis of Resistance Mechanisms to 919TP Infection

In this study, 116 O1 El Tor *V. cholerae* strains (**Supplementary Table S1**) were selected, including 90 toxigenic strains and 26 non-toxigenic strains. These strains were then analyzed with respect to their susceptibility to phage 919TP. Forty-three strains were found to be sensitive to 919TP, of which 36 were toxigenic strains. Subsequently, eight genes from the 919TP phage genome were selected to detect the presence of K139 phage family genes in these bacterial genomes. The eight analyzed genes were not observed in the 43 sensitive strains. Therefore, these strains were deemed not to be lysogenic, and members of the K139 phage family could infect them.

Two potential mechanisms of resistance to 919TP infection were considered in the remaining 73 resistant strains. The first mechanism was dependent on the presence of a temperate K139 genome in the bacterial host. The second mechanism involved presence of mutations in the lipopolysaccharide (LPS) biosynthesis gene cluster (Nesper et al., 2002) associated with the K139 phage receptor (Nesper et al., 2000).

Strains Had Temperate K139 Genome Confer Resistance to 919TP Infection

The 919TP-resistant bacteria that contained the eight genes of the K139 phage family (*orf15, orf19, orf28, orf35, rep, int, cox,* and the K139 family immunity gene *glo*) were analyzed first. The eight genes could be amplified in 50 of the 73 resistant strains, suggesting that these 50 strains may harbor superinfections exclusion as they contain temperate K139 phage family DNA.

These 50 strains were tested to determine if they were capable of producing K139 phage particles. Supernatant samples of 46 of these strains generated plaques in *V. cholerae* N16961 culture, suggesting their capability for the spontaneous release of phage. All the supernatant samples from the 46 strains generating plaques on N16961 culture plates were positive for the four 919TP genes (*orf15, orf19, int,* and *glo*) but negative for the four *V. cholerae* genes (*recA, thyA, rpoA,* and *gyrB*). These results showed that the 46 strains could produce 919TP (or K139 family related) phage (**Supplementary Table S1**). The other four strains, 4070, T21, 2757, and 249, were not observed to release K139-like phage, though K139 family related genomes were present in their genomes (**Supplementary Table S1**). To test whether the genes of the temperate K139 phages in these four strains might still have the possibility to generate progeny phages, we determined the gene transcription of the temperate phages using

qRT-PCR by detecting the transcription of the three phage genes *orf18* (putative major capsid protein), *orf24* (putative tail sheath protein), and *orf28*. Transcription of these genes was observed in the positive control strain 919T, and were also found in the test strains 4070, T21, 2757, and 249, suggesting that the temperate K139 phages in these strains may only generate undetectable progeny phage particles. For these strains, the possibility of defects in the progeny phage assembly at the last step of K139's life cycle should not be excluded.

Further, the binding or adsorption of phage 919TP to temperate K139 phage family *V. cholerae* strains was analyzed by using SYBR gold-stained 919TP particles; both kinds of strains that could and could not produce detectable K139 phage were selected. Fluorescence around the bacterial cells was observed in all test strains in these experiments, indicating the binding of 919TP to these strains.

Receptor Gene Mutations Cause Resistance to 919TP Infection in Strains that Do Not Carry the Genome of the K139 Family

Twenty-three strains were 919TP-resistant but did not contain the temperate K139 phage genome (**Supplementary Table S1**). Receptor sequence mutations might have caused the failure of phage absorption to the bacterial cell, which is one resistance mechanism to phage infection. For nine of these 23 strains, whole genome sequence had been sequenced in our laboratory (Mutreja et al., 2011; Didelot et al., 2015), or the *wbe* gene cluster responsible for synthesis of the K139 phage receptor, LPS, was sequenced in our previous study (Xu et al., 2013). The *wbe* gene clusters of these nine strains, and of two other strains (323 and 2657) sensitive to 919TP, were compared with the 919TP sensitive El Tor strain N16961 (**Table 2**). Mutations in *wbe* gene clusters were found in four of the strains (**Table 2**). One (strain 323) contained only three amino acid residue substitutions in the *wbeW* gene, and this strain was sensitive to 919TP; therefore, such *wbeW* mutations should not alter 919TP phage recognition and infection and was not related to resistance to 919TP. Three of the resistant strains (228, 1888, and 2113) contained concurrent mutations in the genes *manB* and *wbeW* (**Table 2**). These strains had the same *wbeW* mutations as strain 323 that did not affect infection with 919TP. Thus, we cloned and complemented wild-type *manB* into these strains, to determine whether the *manB* mutation was involved in resistance to 919TP infection. Strain 228 became sensitive after complementation with wild-type *manB*. Moreover, CLSM showed no 919TP phage adsorption to cells of strain 228, but when complemented with wild-type *manB*, binding of 919TP to the cells was observed (**Figure 2**). Therefore, the mutations in *manB* resulted in non-binding of 919TP to strain 228, conferring resistance of the bacteria to 919TP. However, sensitivity to 919TP was not restored when strains 1888 and 2113 were complemented with wild-type *manB*. Little or no 919TP adsorption was observed to strains 1888 and 2113 in CLSM experiments (as **Figure 2**, data for strain 2113). These data suggested that in strains 1888 and 2113, there were additional resistance mechanisms to 919TP besides *manB* mutation. Here, we collected the genome data of the nine strains within these 23 919TP-resistant strains as the subgroup samples, to determine the mutations related to this phage resistance. No genome or LPS gene cluster sequence data of the other 14 resistant strains are available right now. It can be deduced that some of these strains may possess these mutations, but more receptor mutations responsible for K139 resistance or even new resistance mechanisms can be found when more resistant strains are included in the study.

V. cholerae Strains that Were Not K139 Lysogenic Isolates and Carried the Same *wbe* Gene Clusters as that of 919TP-Sensitive Strains Were Resistant to the Phage

In the nine 919TP resistant strains for which the sequences of the *wbe* gene cluster are available, six (147, 2255, 2454, 2833,

TABLE 2 | LPS synthesis gene cluster sequencing and associated strains.

Strain ID	Location	Source	Year isolated	*ctxB* gene	Phage production	Phage 919TP plaque formation	K139 genome sequence	LPS synthesis gene cluster mutations
228	Liaoning	Water	2004	–	–	–	–	*manB*[a], *wbeW*[b]
1888	Guangdong	Water	2007	–	–	–	–	*manB*[a], *wbeW*[b]
2113	Guangdong	Water	2006	–	–	–	–	*manB*[a], *wbeW*[b]
147	Guangdong	Patient	2002	+	–	–	–	No mutation
2255	–	Patient	2008	+	–	–	–	No mutation
2454	Hainan	Patient	2005	+	–	–	–	No mutation
2833	Shanghai	–	1979	+	–	–	–	No mutation
2981	Shanghai	Seafood	1965	–	–	–	–	No mutation
X190	Peru	Patient	1991	+	–	–	–	No mutation
323	Liaoning	Water	2005	–	–	+	–	*wbeW*[b]
2657	Yunnan	Patient	1995	+	–	+	–	No mutation

LPS synthesis gene cluster mutations relative to V. cholerae El Tor strain N16961: [a]manB: Amino acid mutation in positions 72 (I to V), 77 (I to L), and 91 (Y to H). [b]wbeW: Amino acid mutation in positions 13 (A to V), 118 (A to V), and 122 (S to A).

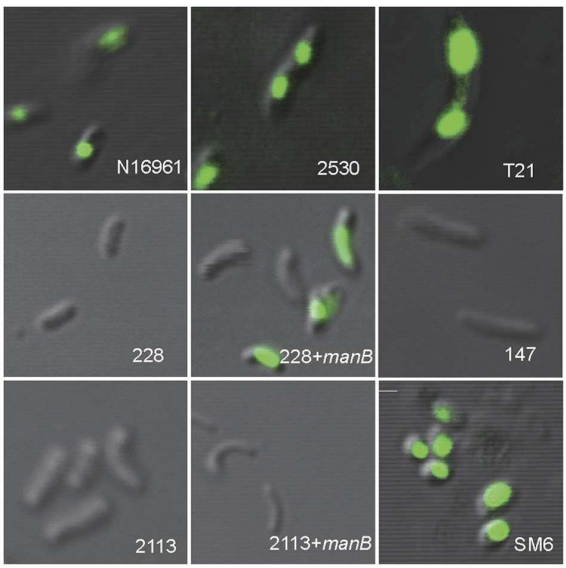

FIGURE 2 | Binding of SYBR gold-labeled phage 919TP to the surfaces of different strains of *V. cholerae* observed by confocal laser scanning microscopy. The strains included N16961 and SM6: sensitive to 919TP; 2530: carrying temperate K139 and capable of producing detectable K139 phages; T21: carrying lysogenic K139 but incapable of producing detectable K139 phage; 228 and 2113: possessing receptor gene mutations; 228+*manB* and 2113+*manB*: complemented with the wild-type *manB*-expressing plasmid and did and did not recover sensitivity to 919TP, respectively; 147: possessing the same *wbe* gene clusters with the sensitive strain 919T but resistant to 919TP.

2981, X190) had almost the same sequence as the 919TP sensitive strain 2657, except for an amino acid residue difference in *WbeT*. This gene was responsible for biotype conversion from serotype Ogawa to Inaba. However, the wild serotype Inaba strains could also be infected by 919TP, suggesting that this mutation on the *wbeT* gene, which was responsible for the serotype conversion from Ogawa to Inaba, may not generate resistance to 919TP. We introduced the plasmid expressing *rfbT* (also named *wbeT*; Liang et al., 2013) into the strains 2454 and 2833 as a representative of these six strains, and both strains recovered the agglutination ability in response to Ogawa anti-serum, indicating that the complemented *rfbT* was active, whereas neither strains showed restored sensitivity to 919TP. Therefore, this mutation in *wbeT* had no role on the resistance to 919TP infection in these strains. These six strains should possess intact LPS which could be recognized by 919TP. Furthermore, two of the strains (147 and 2255) were randomly selected to observe 919TP binding by CLSM. Unexpectedly, no fluorescence was found on the periphery of the cells, suggesting that 919TP did not bind to these strains and that there were factors other than the LPS affecting the binding of 919TP to these strains.

DISCUSSION

Subtyping of bacterial strains below the species level is necessary for variance and clonality studies, and to trace the waves of transmission of pandemics. Exploring the genetic determination of phenotyping can reveal the basic differences among different phenotypes. The Phage-Biotyping Scheme was used to subtype *V. cholerae* O1 El Tor strains based on their phenotype since the 1970s (Wang et al., 2007; Deng et al., 2008). Some genetic mutations of the El Tor strains that were resistant to the typing phages, mainly on the receptor genes (Zhang et al., 2009; Xu et al., 2013, 2014), had been identified. Based on phage resistance and receptor gene mutations, the appearance and spread of a distinct clone was identified in some specific geographical regions (Xu et al., 2014). *V. cholerae* strains in different epidemics or outbreaks also presented different sensitivities to phage 919TP. Therefore in this study, we explored the possible mutations of the 919TP-resistant strains and their other possible strategies by which *V. cholerae* strains resist 919TP infection, in order to reveal their genetic differences related to the 919TP infection compared to sensitive strains. This may help to understand the emergence of resistant strains and even find the genetic markers of these resistant strains.

In this study, the genome of phage 919TP was first sequenced. Unexpectedly, the phage that was in use in our Phage-Biotyping Scheme for many years was a member of the *Vibrio* phage K139 family. K139 was isolated from a *V. cholerae* serogroup O139 strain, but was frequently found in O1 El Tor strains (Reidl and Mekalanos, 1995; Nesper et al., 1999, 2000; Kapfhammer et al., 2002). Although the phage analyzed as part of this study came from *V. cholerae* strains isolated at different times and in different regions, the genome sequences of these phage strains are highly conserved.

Studying the possible strategies that *V. cholerae* strains employ to resist 919TP infection may reveal the genetic variance between the sensitive and resistant strains. As a temperate phage, lysogenicity of the phage in the host strain may confer resistance to the infection by the same phage. Phage K139 uses LPS as its receptor (Nesper et al., 2000), therefore some *wbe* gene mutations in *V. cholerae* strains may also result in resistance by hindering the binding of 919TP. Among the test 919TP resistant strains, most were K139 family lysogenic strains, which may confer superinfection exclusion by 919TP. Such superinfection did not affect the initial step of the K139 phage infection process, i.e., adsorption, but it was probably mediated at a later stage in the infection process through the inhibition of viral replication or integration of the injected genome of the newly infecting phage. Other strains were resistant because of *wbe* gene mutations that cause mutations in the K139 phage receptor thus blocking the adsorption of 919TP onto the bacteria. Therefore, in this study, the genetic characteristics (receptor mutations and temperate K139 in the 919TP-resistant strains) were directly connected to their different phenotypes (resistance to 919TP). Generally, gene mutations were easier to observe, but their biological significance was seldom identified. In the current study, mutations underlying resistance to phage infection were characterized. The biological roles of these mutations were hence identified. Such mutations may serve as markers that complement epidemiological studies

implemented to distinguish and monitor the spread of *V. cholerae* strains and genetic clones.

Serotyping, biochemical tests, phage typing, molecular subtyping and whole genome sequencing are tools for the differentiation of microbiological and genetic characteristics and analysis of the evolution of microorganisms. They were also used in outbreak detection, prevalence description, and source and transmission tracing of pathogens. The Phage-Biotyping Scheme had defined the seventh cholera pandemic strains as limited phage-biotypes (Wang et al., 2007; Deng et al., 2008). The epidemic strains have undergone continuous genetic variations, for instance, mutations to obtain phage resistance conferred the strain with a survival advantage because it can avoid phage-dependent lysis. On other hand, the phage resistance phenotype and its genetic mutation can be used as the markers to distinguish the different strains, to trace the transmission of the epidemic, and to identify the source of the outbreaks, which are the purposes of the Phage-Biotyping Scheme. However, there are still some resistant strains whose resistance could not be explained by the immunity conferred by temperate phage or receptor mutations. Other possible mechanisms that were not experimentally explored in this study include the production of a structured extracellular bacterial cell matrix, degradation of phage nucleic acids through restriction-modification systems or CRISPR/Cas systems in addition to abortive infection systems (Labrie et al., 2010). Further studies are needed to examine other steps of K139 phage infection including specific adsorption, replication, and the initiation of lysis in the K139 infection cycle.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: BK and XS. Performed the experiments: XS, JZ, and JX. Analyzed the data: PD and BP. Contributed reagents/materials/analysis tools: JL, JZ, and JX. Wrote the manuscript: XS. Revised the manuscript: BK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00726

TABLE S1 | The *V. cholerae* O1 El Tor strains used in this study.

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Means to Facilitate the Overcoming of Gastric Juice Barrier by a Therapeutic Staphylococcal Bacteriophage A5/80

Ryszard Międzybrodzki[1,2,3], Marlena Kłak[1,4], Ewa Jończyk-Matysiak[1], Barbara Bubak[1], Anna Wójcik[1], Marta Kaszowska[5], Beata Weber-Dąbrowska[1], Małgorzata Łobocka[6,7] and Andrzej Górski[1,2,3]*

[1] Bacteriophage Laboratory, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [2] Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [3] Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland, [4] Research and Development Center, Regional Specialized Hospital, Wrocław, Poland, [5] Laboratory of Microbial Immunochemistry and Vaccines, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [6] Autonomous Department of Microbial Biology, Faculty of Agriculture and Biology, Warsaw University of Life Sciences – SGGW, Warsaw, Poland, [7] Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

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***Correspondence:**
Ryszard Międzybrodzki
mbrodzki@iitd.pan.wroc.pl

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In this article we compare the efficacy of different pharmacological agents (ranitidine, and omeprazole) to support phage transit from stomach to distal portions of the gastrointestinal tract in rats. We show that a temporal modification of environment in the animal stomach may protect Twort-like therapeutic antistaphylococcal phage A5/80 (from bacteriophage collection of the Hirszfeld Institute of Immunology and Experimental Therapy PAS in Wroclaw, Poland) from the inactivation by gastric juice effectively enough to enable a significant fraction of orally administered A5/80 to pass to the intestine. Interestingly, we found that yogurt may be a relatively strong in enhancing phage transit. Given the immunomodulating activities of phages our data may suggest that phages and yogurt can act synergistically in mediating their probiotic activities and enhancing the effectiveness of oral phage therapy. We also demonstrate that orally applied phages of similar size, morphology, and sensitivity to acidic environment may differ in their translocation into the bloodstream. This was evident in mice in which a therapeutic staphylococcal phage A5/80 reached the blood upon oral administration combined with antacid agent whilst T4 phage was not detected even when applied in 10^3 times higher dose. Our findings also suggest that phage penetration from digestive tract to the blood may be species-specific.

Keywords: bacteriophage, oral administration, gastric juice barrier, phage translocation, antacids, yogurt

INTRODUCTION

Growing antibiotic resistance of bacteria has rejuvenated the interest in using bacteriophages as potential alternatives to antibiotics in the treatment of bacterial infections (Górski et al., 2016). Due to their unique mechanism of antibacterial action phages are able to combat bacteria irrespectively to bacterial antibiotic resistance profiles (Chan et al., 2013, 2016; Cao et al., 2015; Oduor et al., 2016;

Ozkan et al., 2016). Phages use specific receptors at the surface of bacterial cells to recognize targeted bacteria (Rakhuba et al., 2010). Therefore their particular feature is a limited spectrum of bacterial hosts. This ensures the targeting of infecting pathogen and saving beneficial saprophytic flora – an important advantage of phages over antibiotics in the fight with bacterial infections (Abhilash et al., 2008). Despite of the progress in knowledge on the potential application of phage therapy in medical and veterinary practice many basic issues still need to be solved. A crucial factor ensuring the effectiveness of phage therapy is the bioavailability of phages at the infection site. With the exception of external infections or infections in easy accessible body cavities it requires the transit of phage from the site of application to the site where the infecting bacteria reside.

The classification of the phages according to morphology and nucleic acid by the International Committee on Taxonomy of Viruses (ICTV) suggest that differences between them may be extensive. Additionally, even phages belonging to the same genera may differ significantly with respect to their sensitivity to different environmental conditions (Jończyk et al., 2011). However, there is a lack of complex studies which compare bioavailability of different phages (Ackermann, 2003; Leiman et al., 2003; Dąbrowska et al., 2005). Detailed knowledge of these processes (chance for the phage penetration to a site of infection) have significant impact in determining therapeutic recommendations for bacteriophage use. Phage bioavailability after oral administration is one of them.

Oral drug application is one of the most convenient for patients, and it was used for phage application by some clinical centers conducting experimental phage therapy (Weber-Dąbrowska et al., 1987; Chanishvili, 2012; Międzybrodzki et al., 2012; Sarker and Brüssow, 2016). It may be used both as a local application for treatment of intestinal infections as well as a systemic administration for treatment of diseases located outside of the digestive tract (Zelasko et al., 2017). However, the data confirming the possibility of the phage transit through gastrointestinal mucosa in humans are scarce (Weber-Dąbrowska et al., 1987; Pagava et al., 2012).

Results of studies on animals concern single phages and indicate that significant differences between phage ability to penetrate the intestinal wall are possible (Dąbrowska et al., 2005). Geier et al. (1973) studied organ penetration of phage λ in mice. They observed that after oral application of 2×10^{12} phage particles the titer of phage in blood and organs was a few order of magnitude lower than that after intramuscular, intravenous, or intraperitoneal administration, and that the phage could be detected for a shorter time (up to 30 h after *p.o.* administration versus over 50 h after *i.v.* administration). Keller and Engley (1958) showed that after oral administration of 4×10^9 pfu of *Bacillus megaterium 899a* phage to mice the phage could be detected in blood of most of the mice after 5 min and in urine after 30 min. However, they could not confirm the presence of the phage in blood in 14% of mice. Hildebrand and Wolochow (1962) observed that orally administered T1 phage could penetrate into the lymphatic system as it was shown for some bacteria and large proteins but only in a small percentage of tested animals it was able to penetrate into the blood.

Common sensitivity of phages to an acidic environment may significantly reduce phage titers in stomach and the use of antacid during oral phage therapy seems to be convincing solution of this problem (Dąbrowska et al., 2005; Jończyk et al., 2011). The median gastric pH in fasting humans is 1.7 whereas in further parts of alimentary tract it is over 6 (Dressman et al., 1990). This forms physiological barrier to infection but it may also efficiently inactivate certain phages (Jończyk et al., 2011). Therefore the gastric juice barrier is a key factor which may influence phage bioavailability in further parts of digestive tract as well as in blood and body organs.

Oral phage administration is considered as a possible therapeutic option in experimental phage therapy conducted in patients of the Phage Therapy Unit of the HIIET PAS in Wrocław. One of the most frequently used phages is broadly polyvalent *Staphylococcus aureus* phage A5/80 (designated as vB_SauM_A5/80 according to the recommended nomenclature; Kropinski et al., 2009). Its clone of sequenced genome is known as A5W. This tailed phage belongs to the *Kayvirus* genus of the *Spounavirinae* subfamily of myoviruses that was recently separated by ICTV from the previous *Twortlikevirus* genus (Łobocka et al., 2012).[1] In addition to A5/80 the *Kayvirus* genus groups at least 11 phages of highly homologous genomic sequences. A few of this phages, A5/80 among them, have been successfully used in the treatment of staphylococcal infections in humans and animals (Gill et al., 2006; Międzybrodzki et al., 2012; Rose et al., 2014). The A5/80 virion consists of an icosahedral capsid (71.5 nm in diameter which is packed with 146-bp dsDNA molecule, and a 214.5 nm long, contractile tail. It was well characterized at the level of genomic sequence (Łobocka et al., 2012), but its bioavailability after oral administration have not been systematically studied. Therefore, our aim was to verify the effectiveness of different methods of neutralization or reducing stomach juice acidity in overcoming the gastric juice barrier by A5/80 bacteriophage and to test if the application of these methods may improve the transfer of A5/80 through mucosa of the gastrointestinal tract into blood in animal model. *Escherichia coli* model phage T4 was used for comparative purposes in these experiments. It is also a representative of *Myoviridae* family of tailed phages (Leiman et al., 2003). Additionally, its total virion length and head diameter (215 and 85 nm, respectively) are close to those of A5/80.

MATERIALS AND METHODS

Animals

Experiments were performed on female Wistar rats (Wroclaw Medical University) and DBA/1LacJ mice (animal facility of the HIIET PAS under the license from the Jackson Laboratory, USA) housed under standard conditions with food and water *ad libitum*. All experiments were approved by the II Local Ethics Committee in Wrocław, Poland. All oral applications in animals were done into the stomach using a curved feeding needle (Kent Scientific, Torrington, CT, USA).

[1] http://ictvonline.org/proposals/2015.005a-gB.A.v3.Kayvirus.pdf

Phages

T4 phage was purchased from the American Type Culture Collection (ATTC, Rockville, MD, USA). A5/80 phage was obtained from the HIIET PAS therapeutic bacteriophage collection.

Crude phage lysates were prepared according to the modified method of Ślopek et al. (1983). Briefly, phages and the host bacteria were added to peptone water and incubated at 37°C until complete lysis occurred (3–6 h). Phage A5/80 was incubated with cells of *Staphylococcus aureus* 80, and phage T4 was incubated with cells of *E. coli* B strain (standard bacterial hosts for the propagation these phages). Then the suspensions were filtered through a 0.22-μm Millipore filter. Both bacterial strains were from the Polish Collection of Microorganisms (HIIET PAS, Poland).

Testing the Influence of pH on the Phage Survival

Phage-containing lysate (100 μl, 10^7 pfu/ml) was added to 900 μl of saline solutions of different pH: 1.1, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 7.4 (phosphate buffered saline – PBS), and 9.2. Phages were incubated in solutions at 37°C for 60 min. After incubation, each phage suspension was serially 10-fold diluted with PBS for the determination of phage titer.

Testing the Influence of Different Factors Neutralizing or Reducing Stomach Juice Acidity on the Phage Survival *In vitro*

Phage-containing lysate (0.5 ml, 10^7 pfu/ml) was added to 2.0 ml of dihydroxyaluminum sodium carbonate suspension (Alugastrin®, Polfa Łódź SA, Poland), ranitidine hydrochloride syrup (Ranitydyna syrop, 75 mg/5 ml, Sanofi-Syntelabo Sp. z o.o., Poland), 3.2% fat milk (OSM Łowicz, Poland), natural yogurt (Danone Sp. z o.o., Poland) or peptone water as control. Samples were prepared in duplicate and incubated at 37°C for 30 min. After incubation, each phage suspension in buffer was serially 10-fold diluted with peptone water for determination of the phage titer. The change of phage activity was calculated as a percentage of the mean phage titer in both samples of each tested factor relative to the mean phage titer in control samples.

Protocol for Testing the Influence of Different Agents on Overcoming the Gastric Juice Barrier by the Phage

Phage-containing lysate (0.5 ml, 10^7 pfu/ml) was administered orally to rats deprived of food for 24 h before beginning of the experiment. Before phage administration, the rats were given:

- an oral dose of 1.0 ml of 68 mg/ml dihydroxyaluminum sodium carbonate suspension (Alugastrin®, Polfa Łódź SA, Poland) 5–30 min. before phage administration,
- 5–75 mg/kg body weight oral dose of ranitidine hydrochloride syrup (Ranitydyna syrop, 75 mg/5 ml, Sanofi-Syntelabo Sp. z o.o., Poland) 2 h before phage administration,
- 2–50 mg/kg body weight of intraperitoneal ranitidine (Zantac®, for injections, 25 mg/ml, GlaxoSmithKline Export Ltd, United Kingdom) 2 h before phage administration,
- 2.5–10 mg/kg body weight of intraperitoneal omeprazole (Losec®, for injections, 4 mg/ml, AstraZeneca AB, Sweden) 2 h before phage administration,
- 2.0 ml of 3.2% fat milk *per os* (OSM Łowicz, Poland) 1 or10 min. before phage administration,
- 2.0 ml of natural yogurt (Danone Sp. z o.o., Poland) *per os* 1 or 10 min. before phage administration.

Control animals were given the phage lysates only. Rats were sacrificed 30 min. after phage administration, and fragments of duodenum, the middle section of the small intestine, and the caecum were collected.

Testing the Influence of Alugastrin on Gastrointestinal Transit of A5/80 Phage in Rats

Rats were deprived of food for 24 h before beginning of the experiment. They were administered 1.0 ml of Alugastrin *per os* 1–60 min prior to A5/80 phage lysate application (dose: 0.5 ml, 10^8 pfu/ml). They were sacrificed 5–120 min after phage administration and the intestinal contents were collected to determine the phage titer.

Testing the Bioavailability of the Phages after Administration to Rats

Rats were deprived of food for 24 h before beginning of the experiment. In the first experiment A5/80 phage lysate (1.0 ml, 2×10^9 pfu) was applied orally 15 min after Alugastrin (1.0 ml orally or intravenously) and the rats were sacrificed for collection of blood and liver samples before experiment (control), and 1, 2, 4, and 18 h after. In the second experiment Alugastrin was applied 10 min before phage administration (6×10^7 pfu of A5/80 phage, and 4×10^7 pfu of T4 phage in 0.5 ml of the phage lysate) and samples of blood, lymph (from *cisterna chyli*), mesenteric and thoracic lymph nodes, and the middle section of the small intestine were collected. To visualize the lymph to enable its collection the animals received *per os* 1 ml of rape oil 15 min before euthanasia according to Hildebrand and Wolochow (1962). The animals were sacrificed 30 min after phage administration and samples of blood, lymph, mesenteric and thoracic lymph nodes, and small intestine (middle part) were collected to determine the phage titer.

Testing the Influence of Alugastrin on Bioavailability of the Phages after Oral Administration to Mice

The mice were given 0.2 ml of the appropriate phage lysates. Ten minutes before phage administration, they were given an oral dose (0.2 ml) of Alugastrin. Control animals were given the phage lysates only. After 1 h they were sacrificed and heparinized whole blood samples and liver fragments were collected for determination of the phage titer.

Determination of the Phage Titers in Samples

The phage titers both in *in vitro* as well as *in vivo* experiments were determined in duplicate samples using the double-layer agar method according to Adams (1959). Cells of *E. coli* B strain and cells of *S. aureus* 80 strain that were used for the T4 and A5/80 phage propagation were used as indicator strain, respectively. Ten centimeter fragments of duodenum, the middle section of the small intestine were rinsed out with 5 ml of PBS for phage titration in diluted intestine contents. The content of caecum was diluted in PBS in proportion of 100 mg/1 ml. Tissue fragments (spleen, liver, lungs, kidney, brain, and lymph nodes) were homogenized in PBS in proportion of 100 mg tissue per 1 ml of PBS. Lymph was diluted 10–40 times with PBS for phage titer determination. The phage titer in blood was assayed in undiluted blood samples or in samples diluted with PBS when titer was high.

Statistical Analysis

Results are presented as mean phage titer (± standard error of the mean, SE) in the analyzed sample. Differences between the study groups were compared to the control using the non-parametric Mann–Whitney *U*-test (in cases where the number of mice in compared groups was 4 or higher). The differences between the means were considered statistically significant at $p < 0.05$.

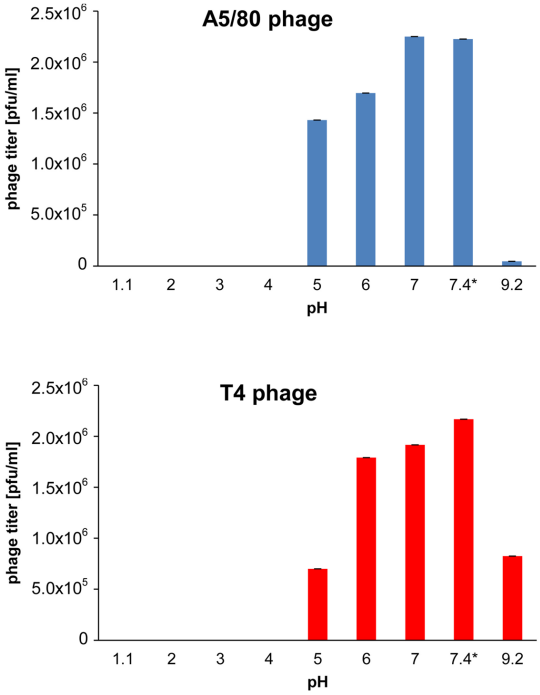

FIGURE 1 | Effect of acidity on phage survival. *Phosphate buffered saline (pH 7.4) was used as control. The phages were incubated in buffers at 37°C for 60 min.

RESULTS

Both, A5/80 and T4, phages used in this study appeared to be sensitive to inactivation at pH below 5, despite, that they differed in the sensitivity to alkaline environment (**Figure 1**). Thus, one can expect that they will be inactivated in a stomach, upon exposure to gastric acid.

Commonly used pharmaceuticals that neutralize the acidity of stomach juice or inhibit its production, when administered orally are dihydroxyaluminum sodium carbonate (Alugastrin), ranitidine hydrochloride (a H_2-receptor antagonist) or omeprazole (a proton pump inhibitor). Additionally, drinking of milk or fermented milk products has been considered as a natural stomach ulcer protective therapy (see e.g., Ippoliti et al., 1976; Modlin, 1995; Elmståhl et al., 1998). Thus, we tested whether certain of these various acidity decreasing agents will not inactivate A5/80 or T4 bacteriophage *in vitro*, when added to the suspensions of these phages (**Table 1**). Milk and yogurt had a negligible effect on the titer of both phages. The influence of Alugastrin on the titer of phage T4 was also negligible, but the titer of phage A5/80 decreased to 40% after 30 min incubation with Alugastrin. Ranitidine hydrochloride syrup decreased the titer of both phages to about 2% or less.

We tested whether the aforementioned agents can increase the ability of A5/80 phage to overcome the inactivating barrier of stomach juice *in vivo* (**Figure 2**). Oral Alugastrin and ranitidine (oral or parenteral) as well as parenteral omeprazole strongly increased the ability of A5/80 phage to overcome gastric juice barrier and pass to the further parts of the gastrointestinal tract in rats. The effect of the highest applied oral versus intravenous dose of ranitidine (75 mg/kg and 50 mg/kg, respectively) on phage titer attained in a small intestine was comparable but more phages were detected in duodenum after *i.v.* application, although a 33% lower dose of the H_2 inhibitor was used. When lower doses of ranitidine were applied we also observed higher phage transit into the intestine in case of intravenous administration (the effect of the lowest *i.v.* dose, 2 mg/kg, was almost 50 times higher than effect of the lowest oral dose – 5 mg/ml). The activity of omeprazole was much higher. When this proton pump inhibitor was applied at a dose of 10 mg/kg it increased the phage titer in a small intestine over six times more as compared to the effect of 50 mg/kg of intravenous ranitidine. At a dose of 2.5 mg/kg the effect of omeprazole was over 8 times stronger than *i.v.* ranitidine at a dose of 2 or 10 mg/kg. When rats were pretreated with 1.0 ml of Alugastrin, the highest phage titer was observed in the small intestine when the phage was applied 5 min. after the administration of Alugastrin and it was over two times higher than the effect of oral ranitidine at a dose of 75 mg/kg. Milk did not improve the intestinal transit of the A5/80 phage significantly (**Figure 2**). Surprisingly, yogurt used just 1 min before the application of the phage increased its titer in the small intestine six times more than the medium dose of oral ranitidine (25 mg/kg). Phage titers in the caecum were usually much lower than those in a small intestine (data not shown).

Further experiments on the influence of Alugastrin on bioavailability of A5/80 phage in gastrointestinal tract of rats showed that the phage reached highest titer in the duodenum and small intestine 30 min after its application (**Figure 3**), and that the optimal time for administration of Alugastrin to increase

TABLE 1 | Comparison of the bioavailability of A5/80 phage at different time points after its oral versus intravenous administration to rats.

Time since the phage administration	Phage titer after oral administration [pfu/ml]				Phage titer after intravenous administration [pfu/ml]			
	Blood		Liver		Blood		Liver	
	n	Mean	*n*	Mean	*n*	Mean	*n*	Mean
1 h	2	0	2	0	3	485[†]	2	419[‡]
2 h	3	0	2	0	1	50	–	–
4 h	2	0	2	0	1	150	1	33
18 h	2	0	2	0	1	5	1	5

All animals received phage at dose of 2 × 10^9 pfu (in 1.0 ml of phage lysate). Alugastrin (1.0 ml) was applied 15 min before the phage administration. [†], min.-max. value: 405–555 pfu. [‡], min.-max. value: 373–465 pfu. No phage was detected neither in blood nor liver in a control group of two animals (non-treated with the phage). N, number of tested animals.

the phage titer in these parts of gastrointestinal tract is its use 1–15 min before the phage administration (**Figure 4**).

We were not able to confirm the presence of active A5/80 phage in blood samples collected during all the above experiments (data not shown). Therefore we conducted more detailed experiments on A5/80 bioavailability after oral administration in rats (**Tables 1**, **2**). Our experiment in which we used a 40 times higher phage dose also did not confirm phage penetration through rat intestinal mucosa into the bloodstream (**Table 1**). Moreover we could not confirm that A5/80 phage was able to penetrate into lymph (**Table 2**). The same results were obtained for T4 phage. Unexpectedly, bioavailability studies done in mice showed contrasting results (**Table 3**). They confirmed that the A5/80 phage was able to penetrate into the bloodstream of mice after its oral administration but it required concomitant use of an antacid (the highest phage titer in blood was observed 60 min after the phage application). In contrast, T4 phage applied at a dose 1000 times higher than A5/80 was detected in blood only in trace amounts (even when the administration of phage was preceded by the administration of Alugastrin).

DISCUSSION

We show here that a temporal modification of environment in the animal stomach may protect therapeutic antistaphylococcal phage A5/80 from the inactivation by gastric juice effectively enough to enable a significant fraction of orally administered A5/80 to pass to the intestine and even to a bloodstream. Our results comparing the efficacy of different pharmacological agents in protecting phage against inactivation by gastric juice revealed that a proton pomp inhibitor, omeprazole was the most efficient followed by a H_2 receptor antagonist, ranitidine, and dihydroxyaluminum sodium carbonate – an agent that is traditionally used in phage treatment conducted at the Phage Therapy Unit in Wroclaw (Międzybrodzki et al., 2012). The data showing the applicability of omeprazole and ranitidine in promoting phage transit to intestinal lumen may be relevant in patients with gastrointestinal disorders where acid production should be under strict control (e.g., peptic inflammation and ulcer).

Although *in vitro* A5/80 phage is nearly completely inactivated upon incubation with hyperosmotic ranitidine syrup or, to a lesser extent, with alkaline Alugastrin (its pH is between 9.9 and 10.2 in a 1:25 suspension[2]), *in vivo* the administration of ranitidine or Alugastrin to animals prior to the oral phage administration significantly increased the number of phages that could pass the stomach juice barrier retaining their activity. A time period separating the administration of ranitidine or Alugastrin from the administration of phage suspension appeared to be an important parameter influencing the phage-protective activity of each of these pharmaceuticals (**Figure 2**), which should be taken into consideration in planning the phage therapy regime whenever phage has to be administered *per os*. To our surprise, yogurt turned out to be relatively efficient in protecting phage from stomach juice, despite that in the case of milk no clear effect was observed. This may be associated with mildly acidic pH of Danone natural yogurt (5.29 according to Çaglar et al., 2011) and with the yogurt buffering activity (Kargul et al., 2007). In *in vivo* studies, the ingestion of yogurt was shown to stabilize the gastric pH for 1 h at the level exceeding 3.5 (Martini et al., 1987). In contrast to that whole milk as well as low-fat milk could cause the increased gastric acid secretion, despite its transient buffering activity (Ippoliti et al., 1976; Khanna and Abraham, 1990; Marotta and Floch, 1991). Taken together, our data suggest that yogurt when added to the current therapeutic protocols of oral phage administration might improve the effectiveness of therapy. This may also have important and therapeutic implications related to suggested immunomodulating activities of phages which may contribute to immunological hemostasis in intestines referred to as probiotic like action of phages (Górski and Weber-Dąbrowska, 2005). Given the well-known probiotic activities of yogurt in gut (reviewed by Adolfsson et al., 2004) and the present data pointing to the so far unknown ability of yogurt to promote phage survival and gastrointestinal passage it suggest that phages and yogurt might act synergistically in mediating their probiotic activities and enhancing the effectiveness of oral phage therapy, for example in the treatment of digestive tract infections. Taking under consideration recent data presented by Przybylski et al. (2015) on the anti-adenoviral activity of T4 phage one may speculate that it might include not only bacterial but also viral infections.

[2]http://www.pharmacopeia.cn/v29240/usp29nf24s0_m26270.html

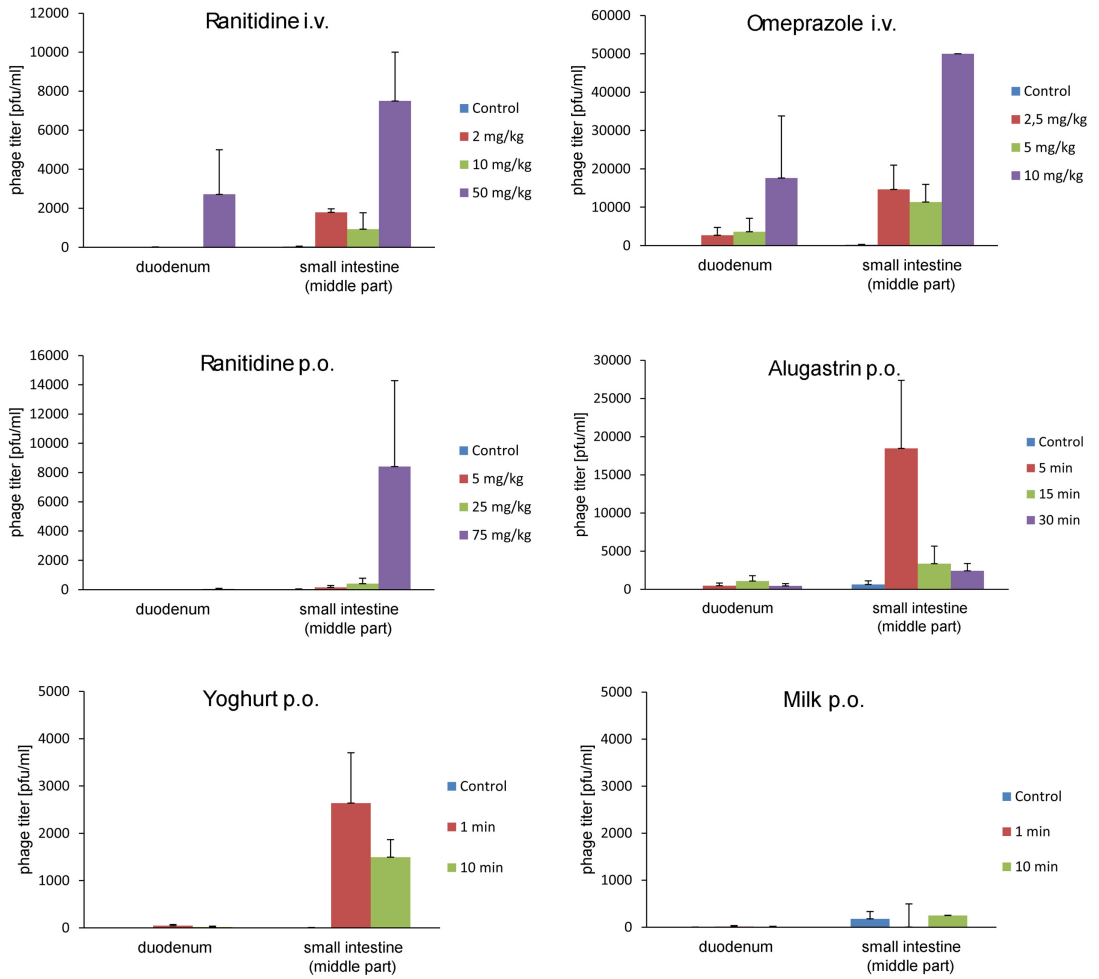

FIGURE 2 | Influence of ranitidine, omeprazole, Alugastrin, yogurt, and milk on A5/80 phage ability to survive in the stomach and to pass into the small intestine. Samples for phage titer determination were taken 30 min after oral administration of 0.5 ml of phage lysate (10^7 pfu/ml). Groups of animals differed depending on a dose of stomach acid inhibitor (ranitidine or omeprazole), or time of the oral application of potential stomach juice neutralizer (1.0 ml of Alugastrin, 2.0 ml of yogurt, or 2.0 ml of milk) before phage administration. Control animals were given the phage lysates only. Shown is mean phage titer ± SE in analyzed samples (n = 2–6).

Potential therapeutic value of this approach requires further studies.

We also aimed to check if overcoming gastric juice barrier by A5/80 phage enables its systemic detection upon oral administration. Because we were not able to detect orally administered A5/80 as well as T4 phage (used here for comparative purposes) in the bloodstream of rats, even when the administration of these phages was combined with antacid agent we performed experiments on mice. Unexpectedly, results for A5/80 and T4 were completely different, despite the similarities in the size and morphology of these phages and their similar sensitivity to acidic environment *in vitro*. This was evident where a staphylococcal A5/80 phage reached the blood upon oral administration combined with gastric acid neutralization whilst T4 phage did not, even though it was applied in 1000-times higher dose. At least a few phage features and non-phage dependent factors could possibly cause this difference. The first one is phage susceptibility to digestive enzymes and bile salts (Ma et al., 2008). However, a sensitivity to these digestive tract components is unlikely in the case of T4 because T4 was able to survive in the small intestine of rats even better than A5/80 (**Table 2**). The second one is a possible phage interaction with bacteria of gut microbiome of tested animals. The A5/80 or T4 adsorption to dead bacteria or to the remnants of their envelopes containing phage receptors will inevitably lead to the irreversible phage inactivation. The adsorption to living bacteria can have several outcomes, with the exception of lysogeny and transduction, as A5/80 and T4 are obligatorily virulent and non-transducing phages (Łobocka et al., 2012, 2014; and references therein). It can be productive leading to a temporal decrease and later to the increase in phage titer, when the progeny of adsorbed phages is released from the infected bacteria. Alternatively, it can be non-productive due to the inability of infected cell to support phage development, to the degradation of injected

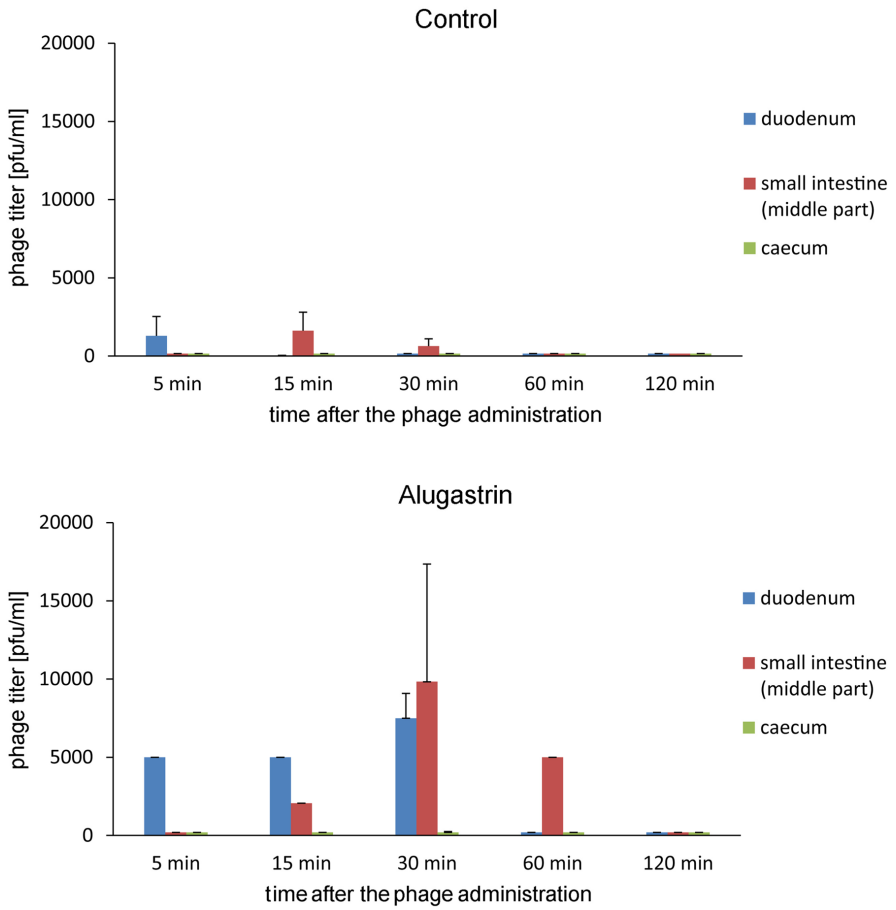

FIGURE 3 | Influence of Alugastrin on A5/80 phage intestinal transit. 1.0 ml of antacid was given to animals 15 min before administration of the phage. Samples of the intestinal contents were collected for phage titer determination at different time points (5–120 min) after oral administration of 0.5 ml of phage lysate (10^8 pfu/ml). Shown is mean phage titer ± SE in analyzed samples (n = 1–4).

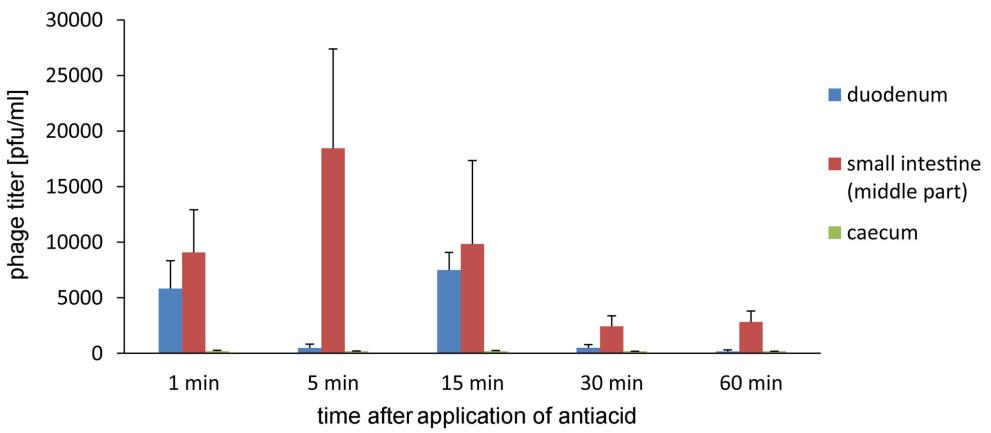

FIGURE 4 | Intestinal transit of A5/80 phage applied orally at different time points (1–60 min) after oral administration of an antacid (1 ml of Alugastrin). Samples of the intestinal contents were collected for phage titer determination 30 min after administration of 0.5 ml of phage lysate (10^8 pfu/ml). Shown is mean phage titer ± SE in analyzed samples (n = 3–4).

TABLE 2 | Orally administered A5/80 and T4 phage penetration into blood, lymph, mesenteric and thoracic lymph nodes, and small intestine (middle part) in rats.

Phage/sample	Phage titer in analyzed samples [pfu/ml]								
	Control			Phage			Phage+Alugastrin		
	n	Mean	*SE*	*n*	Mean	*SE*	*n*	Mean	*SE*
A5/80 phage									
Blood	4	0	0	4	0	0	4	0	0
Lymph	4	0	0	4	0	0	4	0	0
Mesenteric lymph nodes	4	0	0	4	0	0	4	0	0
Thoracic lymph nodes	4	0	0	4	0	0	4	0	0
Small intestine	4	0	0	4	37	37	4	444	271
T4 phage									
Blood	3	0	0	3	0	0	3	0	0
Lymph	2	0	0	2	0	0	1	0	–
Mesenteric lymph nodes	3	0	0	3	0	0	3	6	5
Thoracic lymph nodes	2	0	0	3	0	0	3	5	3
Small intestine	3	12	12	3	1 348	1 116	3	14 667	12 719

Alugastrin was applied 10 min before the phage administration. Animals were euthanized 30 min after application of the phage preparation (0.5 ml per animal). The phage dose was: 6×10^7 pfu of A5/80 phage, and 4×10^7 pfu of T4 phage. 15 min before termination of the experiment all rats (including the control group) received per os 1 ml of rape oil to visualize the lymph. SE, standard error of the mean; n, number of tested animals.

TABLE 3 | Bioavailability of A5/80 and T4 phages in blood and liver after their oral administration to mice.

Phage	Dose [pfu]	Group	Time from the phage application	Phage titer in samples [pfu/ml]					
				Blood			Liver		
				n	Mean	*SE*	*n*	Mean	*SE*
A5/80	5×10^6	Control	15 min	5	217	212	5	20	20
			30 min	6	1	1	6	0	0
			60 min	4	17	17	4	2	2
			120 min	4	11	11	4	1	1
			180 min	4	21	19	4	0	0
		Alugastrin	15 min	4	108	106	4	3	1
			30 min	9	806	482	9	155*	70
			60 min	7	2 783*	936	7	637*	463
			120 min	4	363	363	4	33	32
			180 min	4	28	28	4	10	6
T4	5×10^6	Control	60 min	3	10	8	3	0	0
		Alugastrin	60 min	3	3**	1	3	0**	0
	7×10^9	Control	60 min	3	1	1	3	3	1
		Alugastrin	60 min	3	18**	15	3	66	63

*Alugastrin (0.2 ml) was applied 10 min before the phage administration. Control group received only 0.2 ml of appropriate phage lysate. SE, standard error of the mean; n, number of tested animals. *$p < 0.05$ when compared to control group. **$p < 0.05$ when compared to A5/80 phage and Alugastrin treated group tested after 60 min.*

phage DNA by bacterial restriction enzymes or the CRISPR-Cas immunity system, to the abortive infection mechanisms, or to the bacterial toxin-antitoxin system activation (reviewed by Labrie et al., 2010). In our experiments the decrease of A5/80 or T4 titer upon 15 and 45 min. incubation of each of these phages with the content of mice or rat intestine, did not exceed one order of magnitude (Supplementary Tables 4–6). Moreover, in the case of certain mice we observed an increase in T4 phage titer 45 min. after the phage incubation with the intestine content. These changes are indicative of both the possibility of inactivation of some A5/80 or T4 phages in the intestine as well as the possibility of productive infection of some intestinal bacteria by T4. However, in our opinion they are too small to explain the difference in murine blood titers of A5/80 and T4 applied in 10^3 higher dose than A5/80. We were able to detect bacterial strains susceptible to both phages in the rat intestines (detailed data presented in Supplementary Materials), but were not able to detect them in the intestine of mice. Conceivably, in mice they do not predominate among enterobacteria of the gut microbiome. Another reason of the absence of T4 in blood could be its direct

inactivation in blood – for example by the presence of anti-T4 phage or cross-reacting antibodies (Dąbrowska et al., 2014). Our control experiments showed that 60 min incubation of T4 phage in full blood samples of mice (at 37°C) did not decreased its activity, and that the phage incubation with rat or murine serum resulted only in less than 25% drop in its titer (for details please see Supplementary Tables 2, 3). Therefore, we hypothesize that differences in the ability of A5/80 and T4 to penetrate from the intestine to blood in our experiments might result from different interactions of these phages with intestinal mucus layer and/or intestinal mucosa.

T4-like phages are natural components of mammalian gut as indicated by several cases of their isolation from stool samples (see e.g., Furuse et al., 1983; Kutter et al., 1995; Chibani-Chennoufi et al., 2004). The digestive tract is a natural reservoir of their host bacteria. Evolutionary, these phages could hardly benefit from the passage from the intestinal lumen to a bloodstream. Instead one may expect that they developed strategies to ensure their retention in the digestive tract. The T4 capsid-exposed protein Hoc was shown previously to interact with mammalian organisms (Dąbrowska et al., 2006, 2007) and to bind to mucin glycoproteins (Barr et al., 2013). Recently Barr et al. (2015) demonstrated that the T4 adherence to mucus and Hoc interaction with mucin glycoproteins are responsible for the subdiffusive motion of T4 in the mucus, as compared to the diffusive motion of the T4Δ*hoc* phage. As a result of the subdiffusive motion wild-type T4 could reduce the bacterial colonization of the epithelium 4,000-fold more efficiently than its Δ*hoc* mutant. Possibly, T4 is trapped in the intestinal mucus and thus cannot penetrate further layers of the intestinal barrier. Studies are in progress to find out whether the *hoc* gene deletion will influence the systemic bioavailability of orally administered T4 phage.

In the intestine, a physical barrier between the intestinal lumen, the lamina propria and the mucosal-associated lymphoid tissue is the intestinal epithelium. It is formed by a single layer of cells and mostly contain enterocytes (absorptive epithelial cells), microfold (M) cells (non-absorptive epithelial cells) and goblet cells scattered among them. Mucus secreted by the goblet cells spatially compartmentalizes the bacteria to the lumen (Johansson et al., 2011). The paracellular flux through this layer is limited by tight junctions that form interconnections between the most apical parts of the epithelial cells. The size of particles that can penetrate through tight junctions does not exceed 10 nm (Fihn et al., 2000; Shen et al., 2011), which is too small for the passage of A5/80 and T4 phage. An alternative is a transenterocytic pathway or the M-cell-mediated pathway. The former occurs by endocytosis through the apical enterocyte membrane, followed by intracellular trafficking and exocytosis through the basolateral membrane (reviewed by Yu et al., 2016). However, the endocytosis of A5/80 by enterocytes might require specific receptors as it was shown for certain pathogens that are translocated via this pathway across the intact intestinal barrier. Thus, the more likely way of A5/80 passage through the intestinal barrier is the M-cell-mediated pathway. M cells are specialized in antigen sampling, have a strong transcytotic capacity, and can transport many bacteria and viruses, as well as other antigens from the intestinal lumen to the underlying lymphoid tissues to induce immune responses (Kyd and Cripps, 2008; Gonzalez-Hernandez et al., 2014; Chamcha et al., 2015). Limitations of this pathway are the low proportion of M cells (1%) in the intestinal epithelium, as compared to other cells, and a possibility of capturing the transported bacteria or viruses by macrophages and dendritic cells (Yu et al., 2016).

Our results demonstrating weak, if any, T4 phage ability to translocate through the intestinal mucosa are in agreement with the results of Bruttin and Brüssow (2005) who were not able to detect T4 phages in blood after their oral application to human volunteers. The possibility of passage of orally administered T4-like coliphages (isolated from stool samples of pediatric patients with diarrhea and from environmental water samples) through the intestinal tract of mice was demonstrated previously, but in all these cases the presence of phages was restricted to a gut lumen (Chibani-Chennoufi et al., 2004). In a limited study using three horses, Letarova et al. (2012) also was not able to detect fecal phages in blood although they were detected in faces of animals even over 10^7 pfu/ml. Only Majewska et al. (2015) reported detection of T4 phage in blood after its oral application to mice. In our opinion it could be facilitated by a long-term phage application (the experiment lasted for 100 days), a high phage dose (4×10^{10} pfu/ml of drinking water), as well as by a repeated collection of blood samples which could cause stress and hence could also influence the permeability of the gastrointestinal tract mucosa.

The recent data by Thannesberger et al. (2017) indicate that phages may be detected in large quantities in human urine which suggest that they could translocate from the intestinal tract and migrate to other tissues which has a clear clinical significance and relation to our current data presented in this article. The data of Weber-Dąbrowska et al. (1987) and Pagava et al. (2012) suggesting that in patients on oral phage therapy phages may translocate from intestines to peripheral blood, as well as our data, confirm the value of oral phage application as efficient means of delivering phages to sites of infections and thereby successful therapy in patients with bacterial infections, as described earlier (Międzybrodzki et al., 2012). Interspecies differences in phage translocation reported in this article suggest that this phenomenon should be studied in detail in human clinical trials.

AUTHOR CONTRIBUTIONS

RM: design of the study, performance of experimental part, analysis and interpretation of data for the study, drafting the manuscript (mainly) and revising it critically for important intellectual content, and final approval of the version to be published. MKł, EJ-M, and BB: performance of experiments on animals, drafting the manuscript (partly), and final approval of the version to be published. AW, MKa, and MŁ: performance of some *in vitro* experiments, drafting the manuscript (partly), and final approval of the version to be published. BW-D: production of the phage preparations, drafting the manuscript (partly), and final approval of the version to be published. AG and MŁ:

interpretation of data for the study, revising the manuscript critically for important intellectual content, and final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00467/full#supplementary-material

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Conflict of Interest Statement: BW-D, Mł, AG, and RM have filed patent applications for anti-bacterial use of phages.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A Partially Purified *Acinetobacter baumannii* Phage Preparation Exhibits no Cytotoxicity in 3T3 Mouse Fibroblast Cells

*Alexandra E. Henein[1†], Geoffrey W. Hanlon[1], Callum J. Cooper[2], Stephen P. Denyer[1] and Jean-Yves Maillard[3]**

[1] School of Pharmacy and Biomolecular Sciences, Brighton University, Brighton, UK, [2] Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden, [3] Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK

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***Correspondence:**
Jean-Yves Maillard
maillardj@cardiff.ac.uk

[†] *Deceased*

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A surge in the level and scale of antibiotic resistance has prompted renewed interest in the application of bacteriophages to treat bacterial infections. However, concerns still exist over their efficacy and safety. *Acinetobacter baumannii* phage BS46, a member of the family *Myoviridae,* has previously been shown to be effective in murine models. The cytotoxic effect of this phage was evaluated in mouse fibroblast 3T3 cells using four different assays: trypan blue; staining with Hoechst and propidium iodide; lactate dehydrogenase release; and the MTS assay. The addition of phage concentrations up to 2×10^9 pfu/mL showed little to no impact on the viability of 3T3 cells after 24 h exposure using the different assays. This study demonstrates that phage BS46 is non-cytotoxic to 3T3 cells using four different assays and that appropriate quality assurance protocols for phage therapeutics are required.

Keywords: bacteriophage, cytotoxicity, *Acinetobacter baumannii*, lactate dehydrogenase, MTS assay, trypan blue, hoechst stain, propidium iodide

INTRODUCTION

There is a resurgence of interest in phage to treat multidrug resistant infections in humans, with some bacteriophage (phage) products entering clinical trials (Rhoads et al., 2009; Wright et al., 2009) or in animal testing (Biswas et al., 2002; Morello et al., 2011). Although the use of phages within a clinical setting dates back to the early part of the 20th Century (Kutter et al., 2010), their use has been primarily confined to the former Soviet Union and Eastern Europe, in part due to concerns about safety and efficacy (Hanlon, 2007). Small numbers of phage preparations have been approved by the U.S. Food & Drug Administration for use as food additives within the food industry for the control of *Listeria monocytogenes* (Bren, 2007) and within the European Union (Anonymous, 2012). The small amount of human clinical trial data that exists in currently available literature has not demonstrated toxicity issues (Merabishvili et al., 2009). However, the potential to induce an immune response regardless of the level of preparation purity is of major concern to those seeking to utilize phage therapy clinically. There are a number of assays available to measure cell cytotoxicity. These often differ in the parameters they measure and have been shown to exhibit different levels of sensitivity (Fotakis and Timbrell, 2006). No standard assay for determining the cytotoxic effect of phage has been reported.

Acinetobacter baumannii is an opportunistic human pathogen causing a wide range of infections including wound, urinary tract infections (Antunes et al., 2014) and ventilator-acquired pneumonia (Qureshi et al., 2015). *A. baumannii* is an important multidrug resistant microorganism (Cai et al., 2012; Zarrilli et al., 2013; Antunes et al., 2014), with resistance to colistin being recently reported (Qureshi et al., 2015). The use of phages against this important pathogen is an exciting prospect, but for application in humans, particularly for wound infections, their cytotoxicity needs to be evaluated. Here the *A. baumannii* phage BS46, a member of the *Myoviridae* family was used. It has previously been shown to provide a protective effect in mice when challenged with up to five times the LD_{50} (10^8 cfu) of *A. baumannii* (Soothill, 1992).

The current investigation sought to determine the cytotoxic effects of a single *A. baumannii* phage and to evaluate different methods of determining cytotoxic effect following the addition of bacteriophage.

MATERIALS AND METHODS

All chemicals and reagents were obtained from Fisher Scientific (Loughborough, UK) or Sigma–Aldrich (Gillingham, UK) unless otherwise stated in the text.

Sterile Lambda (λ) buffer was prepared by the addition of 2.5 mL 2% (w/v) gelatin to 6 mL 1 M Tris base at pH 7.2. To this 2.5 g $MgSO_4.7H_20$ was added and the resulting suspension made up to 1 L with dH_2O. The resulting solution was then autoclaved at 121°C for 15 min.

Routine Culture of Bacterial and Bacteriophage Strains

Acinetobacter baumannii HER1401 and phage BS46 (Soothill, 1992; Ackermann et al., 1994) were obtained from The Felix d'Hérelle Reference Center for Bacterial Viruses, GREB, Faculté de Médecine Dentaire, Université Laval, Canada.

Acinetobacter baumannii HER1401 cultured in Tryptone Soy Broth (TSB; Oxoid, Basingstoke, UK) for 18 h at 37°C under 120 rpm agitation was used to inoculate 100 mL TSB. That was then incubated for 3–5 h at 37°C under 120 rpm agitation until an OD_{600} of 0.5 ($\sim 10^8$ cfu/mL) was reached. Bacterial suspensions were then infected with 1 mL phage suspension ($\sim 10^6$ pfu/mL), statically incubated at 37°C for 15 min and then incubated for a further 18 h at 37°C and 120 rpm.

Following incubation 10 mL chloroform was added and then the suspensions were incubated at 37°C, 120 rpm for 10 min prior to centrifugation at 2500 × *g* for 10 min. The resulting supernatant was then passed through a 0.45 μm syringe filter (Millipore, UK) to produce a crude lysate.

Production of Concentrated Purified Phage

Prior to use, crude lysates were enumerated and assessed for viability using the agar overlay method (Adams, 1959). To each crude lysate, sodium chloride was added to give a 1 M final concentration, stored on ice for 1 h and centrifuged at 11000 × *g* for 10 min at 4°C. Following centrifugation, 10% (w/v) polyethylene glycol 8000 (PEG 8000) was added to the supernatant and stored at 4°C for 18 h.

Suspensions were centrifuged at 11000 × *g* for 10 min at 4°C, the supernatant discarded, the pellet re-suspended in 11 mL λ buffer and 1 mL chloroform. The organic and aqueous phases were separated by centrifugation at 3000 × *g* for 15 min at 4°C. The organic phase was discarded and the aqueous phase made up to 50 mL with λ buffer and passed twice through 0.45 μm filters. Purified phage preparations were diluted in λ buffer to provide a working suspension of 8×10^9 pfu/mL and enumerated using the agar overlay method as previously described (Adams, 1959) and stored in sterile glass containers at 2–8°C.

Prior to use in cytotoxicity experiments, phages were diluted in λ buffer to yield 2×10^9, 2×10^8, and 2×10^7 pfu/mL and suspensions equilibrated to room temperature.

Cell Culture

Acinetobacter baumannii is a known bacterial pathogen associated with wounds. The embryonic Swiss albino mouse fibroblast cell line '3T3(+3)' ECACC No. 89022402 was used here as a model since fibroblasts are associated with wound healing. 3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1 g/L glucose, L-glutamine and sodium bicarbonate, 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin and streptomycin. All cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere.

Analysis of Bacteriophage Cytotoxicity in 3T3 Cells

Preparation of Cells for Use in Cytotoxicity Experiments

Working culture plates were produced by the addition of 5000, 10000, 15000, or 2×10^5 3T3 cells (depending on the assay type) to wells of sterile Nunclon surface 24-well cell culture plates (Nunc Denmark) into a total volume of 1 mL pre-warmed supplemented DMEM and incubated for 24 h at 37°C in 95% air: 5% CO_2 in a humidified atmosphere.

In order to remove mouse fibroblast 3T3 cells from 24 well plates, DMEM was removed, 200 μL of trypsin added to each well and plates incubated for 3–5 min at 37°C in a 95% air: 5% CO_2 atmosphere. Cell suspensions were centrifuged at 400 × *g* for 5 min, the supernatant discarded and the pellet re-suspended in 1 mL fresh DMEM.

Trypan Blue Exclusion Assay

3T3 cell suspensions and 50 μL purified phage in experimental wells ($n = 6$) were prepared and incubated for 24, 48, and 72 h as before. λ buffer was used as a negative control.

Following trypsinisation, cell viability was assessed using a Trypan blue assay (Strober, 2001). In brief, 20 μL of cell suspension was added to 4 μL 0.4% (w/v) trypan blue solution in phosphate buffered saline (Sigma–Aldrich, Gillingham, UK) and the total number of cells immediately quantified from five squares of a haemocytometer at 100 × magnification using an inverted light microscope (Wilovert standard; Hund Wetzlar, Germany).

If the cell density was too high then cells were diluted 1:10 in DMEM prior to imaging. Unstained cells were considered to be alive while blue stained cells were considered to be dead. In all cases, samples from each well were read in quintuplicate on two different occasions.

Hoechst and Propidium Iodide Staining for Cell Apoptosis

BS46 phages were inoculated to growing 3T3 cells as described above. λ buffer was used as a negative control.

Viable and apoptotic cells were quantified at multiple time points ($t = 24$, 48, 72 h) after phage addition using a modified propidium iodide (PI) staining method (Belloc et al., 1994). In brief, 900 μL of DMEM was mixed with 50 μL of a 1 mg/mL (in 10 mL demineralized water) PI solution and 50 μL of bisbenzimide. Wells were stained with PI and six random fields of view counted using an Axiovert 25 inverted fluorescence microscope (Karl Zeiss Ltd., Welwyn Garden City, UK) with a 420 nm filter. Viable cells were identified by uniform blue fluorescence and apoptotic cells by their fragmented nuclei with either blue or pink fluorescence.

Lactate Dehydrogenase (LDH) Release

Lactate Dehydrogenase (LDH) values were determined using the CytoTox 96 Non-Radioactive cytotoxicity assay (Promega, Southampton, UK) according to the manufacturer's instructions. Absorbance at 492 nm was determined in an automatic Titertek Multiskan Plus MKII plate reader (LabX, Midland, ON, Canada), at 492 nm. Absorbance values were adjusted to compensate for the contribution of DMEM and the percentage cytotoxicity calculated as below;

% Cytotoxicity = [Experimental LDH release/Maximum LDH release] × 100.

Cell Viability Using an MTS Assay

Fifty micro liter of purified phage suspension were added to the experimental wells and incubated for 24, 48, and 72 h as described above. Negative controls containing 50 μL λ buffer and untreated cells were also performed. The number of viable cells was determined using the CellTiter 96 aqueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorbance at 492 nm was determined on an automatic Titertek Multiskan Plus MKII plate reader (LabX, Midland, Canada) and expressed as a percentage of non-treated controls to calculate the percentage proliferation status (Carmichael et al., 1987).

Statistical Analysis

All experiments were performed in triplicate on different days unless otherwise stated in the text.

Trypan Blue Exclusion Test

Data were statistically analyzed using a Kruskal–Wallis One way analysis of variance due to the data being non-normally distributed.

Apoptotic Assay

Data were statistically treated using a Univariate General linear model and Levene's test.

MTS Assay

One-way between groups ANOVA with Tukey's *post hoc* test was used to compare cell viability as percentage of controls.

RESULTS

Trypan Blue Assay

The initial concentration of phage showed a non-significant impact ($p > 0.05$) on the viability of 3T3 cells after 24 h exposure (**Figure 1**) with 2×10^8 pfu/mL exhibiting the highest reduction in viability (∼10%). Seeding density (5×10^3 or 2×10^5 cells/well) also had no significant effect on the viability of cells following 24 h exposure to phage ($p > 0.1$ Tukey's and Kruskal–Wallis).

Hoechst and Propidium Iodide Staining

Statistically significant reductions in the number of viable cells were observed following 72 h incubation with either 2×10^7 and 2×10^8 pfu/mL compared to untreated controls (**Figure 2**; $p < 0.001$). Incubation time had no significant impact on cell viability ($p = 0.418$).

LDH Release

Phage addition at varying concentrations resulted in small but not statistically significant increases in cell death compared to untreated samples (between 5 and 10% reduction; **Figure 3**) with the exception of 2×10^9 pfu/mL at 24 h ($p < 0.001$).

MTS Assay

Following 24 h exposure to different concentrations of phages (2×10^9, 2×10^8, or 2×10^7 pfu/mL) the number of viable cells appeared to increase at higher initial seeding densities (10000 and 15000 cells/well) compared to controls treated with λ buffer only (**Figure 4A**). At 48 h incubation there was no significant difference between phage samples and λ buffer (**Figure 4B**; $p > 0.5$). After 72 h cells exposed to phages cell viability increased significantly compared to the controls. Cells seeded at 10000 cells/well remained more viable (**Figure 4C**; $p < 0.05$) when exposed to 2×10^9 and 2×10^8 pfu/mL phage, compared to λ buffer and untreated (DMEM only) cells.

DISCUSSION

Bacterial cell products can have both direct and indirect cytotoxic effects on cells in the stationary phase (Perfetto et al., 2003), particularly through the action of bacterial endotoxins which affect the expression of adhesion molecules, inflammatory responses (Tang et al., 2011), cytokine release (Perfetto et al., 2003; Tardif et al., 2004), or the release of bacterial toxins (Los et al., 2013). However, little information exists in the current

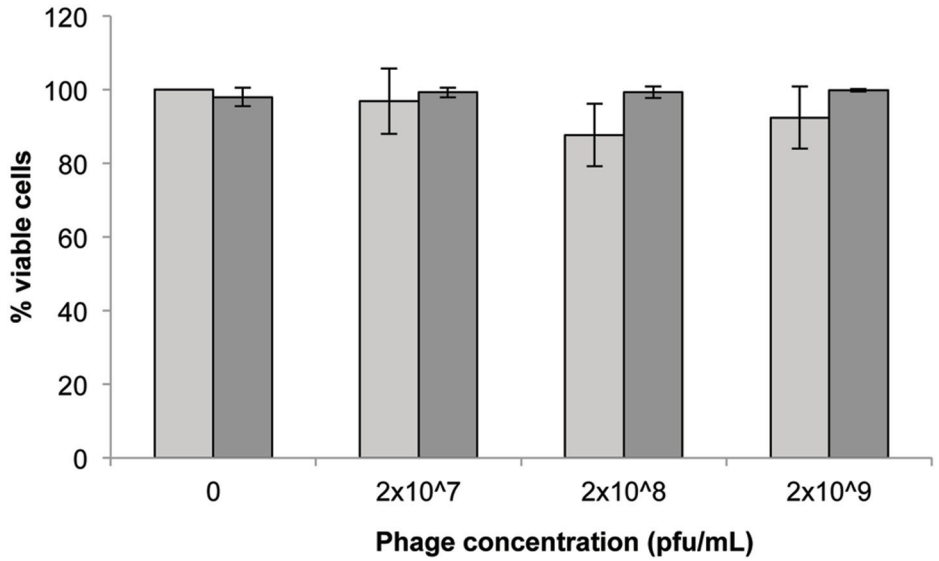

FIGURE 1 | 3T3 mouse fibroblast cell viability following 24 h exposure to *Acinetobacter* phage BS46 in DMEM by trypan blue exclusion assay. ■: 5×10^3 cells/well; ■: 2×10^5 cells/well. Data are the mean of 3 replicates ± SD.

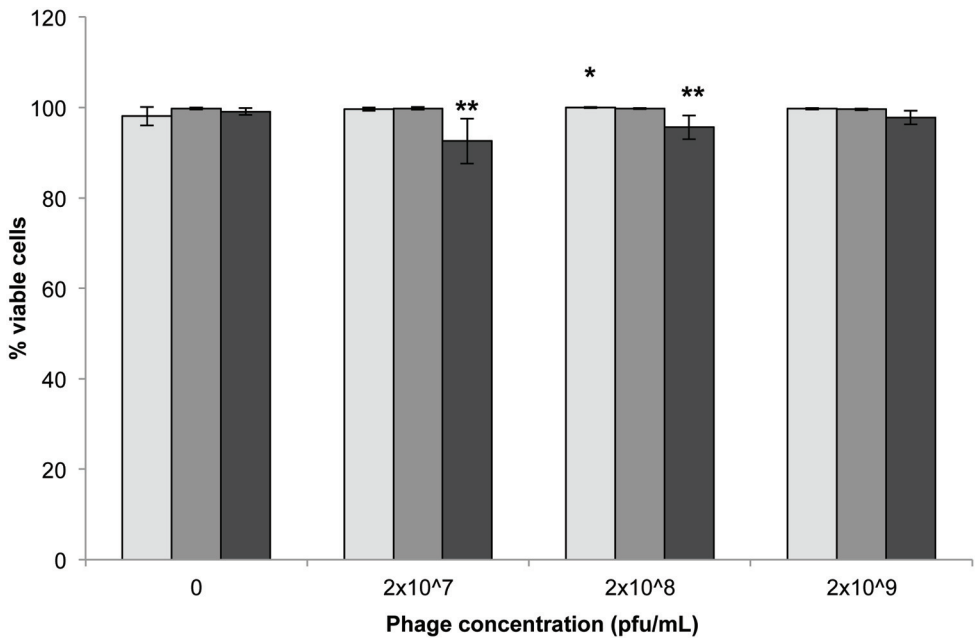

FIGURE 2 | 3T3 mouse fibroblast cell viability following exposure to *Acinetobacter* phage BS46 by Hoechst propidium iodide staining. ■: 24, ■: 48 and ■: 72 h. Data shown are the mean of 3 replicates ± SD. *$p < 0.05$ compared to control, **$p < 0.001$ compared to controls and highest phage concentration (2×10^9 pfu/mL) at 72 h.

literature on the effects of the direct addition of therapeutic bacteriophages on immortalized cell lines such as 3T3 fibroblasts.

In the current investigation, a partially purified *A. baumannii* phage preparation was not cytotoxic to 3T3 mouse fibroblast cell line, although some assay dependent differences in viability were observed. No statistically significant differences were seen between 3T3 cells treated with phage and untreated controls, corresponding with similar investigations performed by Merabishvili et al. (2009) against human neonatal foreskin keratinocytes when assessed by trypan blue exclusion. While patient derived cells will offer the closest analog to whole organism testing *in vitro*, the use of such cell types comes with associated cost and ethical considerations as well as increased variation between multiple donors. Although no substitute

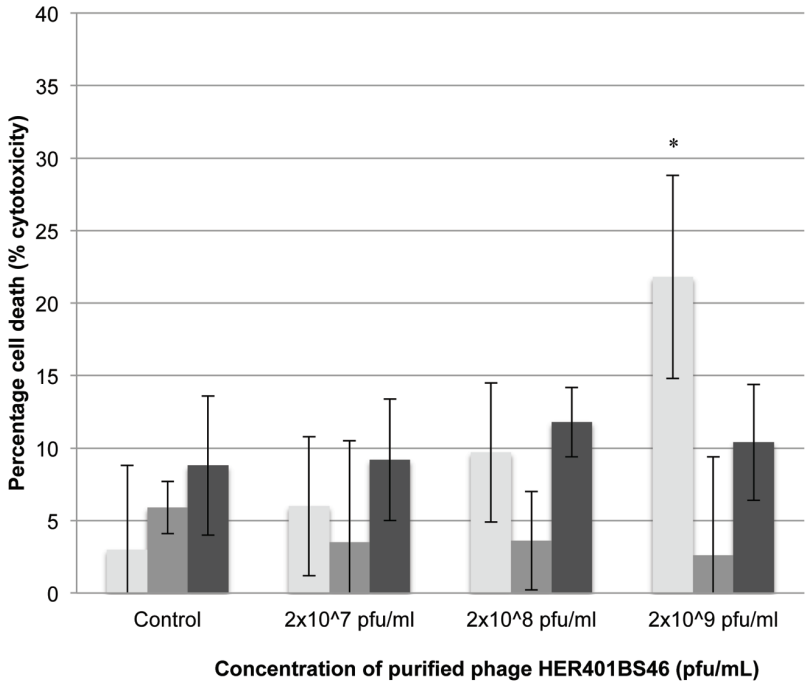

FIGURE 3 | LDH release of 3T3 mouse fibroblast cells following exposure to *Acinetobacter* phage BS46. *$p < 0.001$ compared to the control. ■: 24, ■: 48, and ■: 72 h. Data shown are the mean of 3 replicates ± SD.

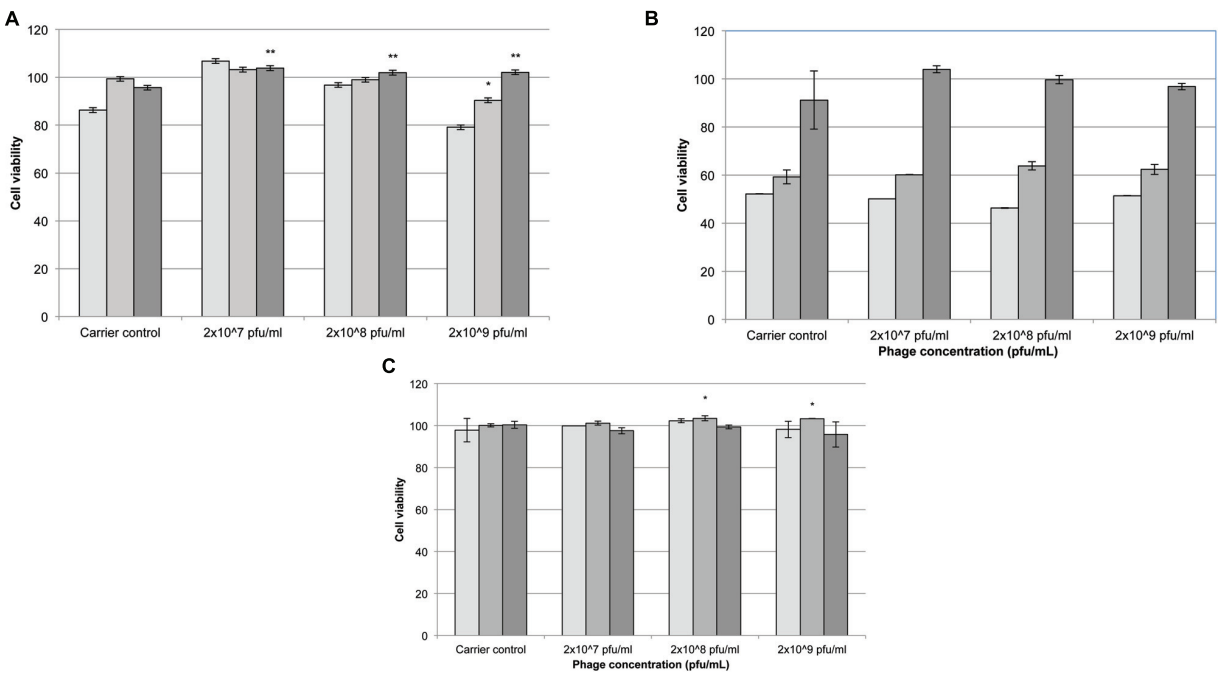

FIGURE 4 | The effect of *Acinetobacter* phage BS46 on MTS formation of 3T3 cells. Percentage cell viability was calculated from the untreated cells which represent 100% viability. Carrier control corresponds to cells treated with λ buffer. **(A)**: 24 h, **(B)**: 48 h, and **(C)**: 72 h incubation. ■ 5000 cells/well, ■ 10000 cells/well and ■ 15000 cells/well. *$p < 0.05$, compared to 2×10^7 pfu/mL; **$p < 0.05$ compared to λ buffer. Data are the mean of 3 replicates ± SD.

for *in vivo* testing, the use of immortalized cell lines would decrease the overall research cost of preclinical studies and enable standardization between laboratories, allowing for better comparative testing to be performed. In addition fibroblasts were used in an attempt to understand the effect of phage on skin or wounds, recognizing that *A. baumannii* is an important wound pathogen.

Although no cytotoxic effect was observed with all the assays tested, the assessment of cellular viability with the MTS assay suggested that phages provided a positive effect on cell viability. This observation supports that of Chung et al. (2010) who reported that genetically engineered M13 bacteriophage improved cell attachment under specific experimental conditions. Although no direct cytotoxic effect was observed, the addition of phages has been shown to induce pro-inflammatory cytokines such as IL-10 and IFN-γ (Dąbrowska et al., 2014; Park et al., 2014). Majewska et al. (2015) reported a weak antibody production in mice following T4 phages ingestion. However, this is dependent on phage type and the protein composition of individual phages (Dąbrowska et al., 2014) and would be of particular importance in non-topical applications that would result in the increased exposure of phages to the immune system (Hodyra-Stefaniak et al., 2015). Here we have used a partially purified phage and it is possible that bacterial debris were present in the preparation. However, the phage preparation used here did not show any cytotoxicity overall. The endotoxin level of any phage product would need to be measured for regulatory purposes before commercialization. A number of methods to reduce endotoxin levels have been described: a single round of PEG precipitation and centrifugation has been shown to remove up to 88% of endotoxins from a bacteriophage preparation (Branston et al., 2015). However, additional purification stages such as ultracentrifugation in a CsCl gradient or chromatographic methods will further reduce impurities (Boratyński et al., 2004).

The current investigation sought to assess the cytotoxicity of a purified preparation of *A. baumannii* phage BS46 using four separate methods. Some discrepancies in the results were observed, notably at high phage concentrations, with the PI and MTS assay showing some small but statistically significant reduction in cell number and the LDH assay showing no significant differences in cell viability. The LDH assay presented the highest variability in results as indicated by the large error bars. It measures the activity of the oxidoreductase of LDH, a stable enzyme, released from damaged cells in the culture medium. Variability in activity, which has been reported in other applications (Bopp and Lettieri, 2008), may be cause by differences in cell seeding. Such variability in results was not noted with the other assays performed. Both the Trypan blue exclusion assay and the Hoechst and PI assays require microscope based analysis which may limit their usefulness although high throughput protocol could potentially be increased using flow cytometry. The MTS assay gives indirect measurement of viability [requires metabolism of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] to formazan and not a measurement of cell damage. This indirect measurement based on cell metabolism may explain why the MTS assay was the only one that showed an increased in cell viability. For developing a product for phage therapy, for which a high number of samples need to be processed, the high throughput trypan blue assay may be the most practical protocol to use. It is thus important to consider the method used to assess the cytotoxic effects of phage preparations and highlights the pressing need for standardized phage testing protocols.

The purified phage suspension exhibited little toxicity and this result is encouraging for the treatment of topical infections with phages. For non-topical applications, the assessment of cytotoxicity could be complemented with the determination of immune-stimulatory capacity (Merabishvili et al., 2009; Szermer-Olearnik and Boratyński, 2015).

AUTHOR CONTRIBUTIONS

Conceived and designed experiments: AH, GH, SD, J-YM. Performed experiments AH. Analyzed data: AH, GH, SD, J-YM. Wrote the manuscript: CC, J-YM, GH, SD.

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Morphologically Distinct *Escherichia coli* Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release *In Vitro*

*Mohammadali Khan Mirzaei, Yeneneh Haileselassie, Marit Navis, Callum Cooper, Eva Sverremark-Ekström and Anders S. Nilsson**

Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

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****Correspondence:***
Anders S. Nilsson
anders.s.nilsson@su.se.

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Due to a global increase in the range and number of infections caused by multi-resistant bacteria, phage therapy is currently experiencing a resurgence of interest. However, there are a number of well-known concerns over the use of phages to treat bacterial infections. In order to address concerns over safety and the poorly understood pharmacokinetics of phages and their associated cocktails, immunological characterization is required. In the current investigation, the immunogenicity of four distinct phages (taken from the main families that comprise the *Caudovirales* order) and their interaction with donor derived peripheral blood mononuclear cells and immortalized cell lines (HT-29 and Caco-2 intestinal epithelial cells) were investigated using standard immunological techniques. When exposed to high phage concentrations (10^9 PFU/well), cytokine driven inflammatory responses were induced from all cell types. Although phages appeared to inhibit the growth of intestinal epithelial cell lines, they also appear to be non-cytotoxic. Despite co-incubation with different cell types, phages maintained a high killing efficiency, reducing extended-spectrum beta-lactamase-producing *Escherichia coli* numbers by 1–4 $\log_{10}$ compared to untreated controls. When provided with a suitable bacterial host, phages were also able to actively reproduce in the presence of human cells resulting in an approximately 2 $\log_{10}$ increase in phage titer compared to the initial inoculum. Through an increased understanding of the complex pharmacokinetics of phages, it may be possible to address some of the safety concerns surrounding phage preparations prior to creating new therapeutic strategies.

Keywords: pharmacokinetics, phage therapy, cytokines, immune response, multi-resistant bacteria

INTRODUCTION

Since their initial discovery, bacteriophages have been used as antibacterial agents in a number of specialist centers in Eastern Europe. However, their initial clinical successes were later overshadowed by the efficiency and increased use of antibiotics in Western medicine. Nonetheless, the global spread of multi-drug resistant bacteria has revitalized research into phage biology and its applications, as new strategies are sought to combat antibiotic resistant bacterial infections (Kutter et al., 2010; Nilsson, 2014). Although phage therapy possesses a number of advantages

over antibiotics, including the relative ease of isolation of new phages compared to finding new antibiotics as well as ability to self-replicate, concerns over efficacy and safety have also hindered their adoption in the West for use in humans (Abedon, 2010, 2011).

In order to successfully combat a bacterial infection, phages must first reach the site of infection in sufficiently high concentration to be able to reproduce to an extent that eventually all or almost all bacteria will become infected. This requires not only high initial doses of phages but also that the phages need to persist in the presence of, or where possible evade, the neutralizing effect of the patients immune system. Although initially believed to be non-immunogenic and non-toxigenic (Adams, 1959) recent studies have shown that high concentrations of phage can induce pro-inflammatory responses, while long term exposure to phages could induce an antibody response in which the spleen plays a key role in phage clearance, which has been demonstrated to be the case for phages T4 and T7 (Dabrowska et al., 2014; Majewska et al., 2015; Hodyra-Stefaniak et al., 2015). Among the safety concerns surrounding the use of phages to combat bacterial infections is also the potential to induce toxic shock caused by the release of lipopolysaccharide (LPS) as a result of bacterial lysis. The injection of low doses of such endotoxins to the human body has been shown to trigger inflammatory responses, and the endotoxin content of pharmaceutical preparations is therefore tightly controlled during manufacture (Merabishvili et al., 2009; Neyen and Lemaitre, 2015). In order to be of clinical use, phage preparations would also have to conform to varying regulatory standards that are contingent on route of administration, prior to approval for use.

In the current study, the cytokine response of human intestinal epithelial cells (IEC) and immune cells to four distinct phages was evaluated in addition to the mitogenic or cytotoxic effects of the phages on these cells.

MATERIALS AND METHODS

All experiments in the current study were performed in triplicate unless otherwise stated in the text.

Bacterial and Bacteriophage Culture

Escherichia coli strains (ECOR10, ECOR32, ECOR57, and ECOR63) were obtained from the *E. coli* reference collection (ECOR; Ochman and Selander, 1984), and were routinely cultured in lysogeny broth (LB) at 37°C with shaking at 120 RPM.

Escherichia coli phages SU10 (*Podoviridae*), SU32 (*Siphoviridae*), SU57 (*Siphoviridae*), and SU63 (*Myoviridae*) were isolated from the Käppala Wastewater treatment works, Lidingö, Stockholm county, Sweden, and have been characterized elsewhere (Khan Mirzaei et al., 2014; Khan Mirzaei and Nilsson, 2015). Phages were routinely cultured in LB with appropriate hosts (ECOR10, ECOR32, ECOR57, and ECOR63) prior to purification in PEG-8000 and CsCl gradient centrifugation as previously described (Villafane, 2009). Phages were enumerated using the agar overlay method as previously described (Khan Mirzaei and Nilsson, 2015). Extended-spectrum beta-lactamase producing *E. coli* strains, here denoted ESBL-32 (strain 07RAFM-ECO-32) and ESBL-198 (strain 07RAFM-ECO-198), were grown in RPMI-1640 complete medium (Invitrogen, Sweden) supplemented with 10%(v/v) heat inactivated fetal calf serum (FCS; Invitrogen), 1%(v/v), penicillin–streptomycin (PEST; Thermo Scientific, Logan, UT, USA), 2%(v/v) L-glutamine and 4%(v/v) HEPES (HyClone Laboratories, Inc, South Logan, UT, USA). ESBL-198 was used for the infection of SU32, SU57, and SU63 while ESBL-32 was used due to the limited host range of SU10.

Preparation of Bacterial Debris

Bacterial debris was prepared from a 1.5 L overnight culture, centrifuged at 11000 × g for 20 min and re-suspended in 20 mL of phosphate buffered saline (PBS) then sonicated with 80% power for 15 min in a VCX130 Vibra-Cell (Sonics & Materials Inc., Newtown, CT, USA) to break up bacterial cells. Bacterial debris samples were subjected to CsCl purification as previously described (Villafane, 2009). In order to confirm the efficacy of debris removal, samples were taken from the same point in the ultracentrifuge tube as phage stocks.

Stimulation of HT-29 and Caco-2 Cell Lines by Purified Bacteriophages

HT-29 (HTB-38) and Caco-2 (HTB-37) were cultured at 37°C in a 5% CO_2 atmosphere in McCoy's 5A (modified) medium (ATCC or HyClone Laboratories Inc.) and in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Inc.), respectively. Both media were supplemented with 10%(v/v) FCS and 1%(v/v) PEST. To maintain exponential growth phase, cells were treated with trypsin-EDTA (Invitrogen) and sub-cultured before reaching confluence (Haileselassie et al., 2013).

In order to provide stimulation, HT-29 and Caco-2 cells were trypsinated, washed in fresh medium and seeded at a final concentration of 2×10^5 cells/well for cytokine analysis in 48 well plates (Costar, Cambridge, UK) and 5×10^4 cells/well in flat-bottomed 96-well plates (Sarstedt Inc., Newton, NC, USA) and grown over night at 37°C in a 5% CO_2 atmosphere. Following incubation, both Caco-2 and HT-29 cells were stimulated as follows: (1) addition of phages alone (10^9 PFU/well), (2) addition of bacteria alone (10^5 CFU/well), (3) simultaneous addition of phages and the host bacteria (10^9 PFU/well and 10^5 CFU/well), and (4) complete media only. Cells were incubated for 8 h for cytokine analysis and 72 h for 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) analysis at 37°C in 5% CO_2. Supernatants from IEC stimulations were collected and frozen at −20°C until cytokine analyses and the cells were subjected to MTT analysis.

Human Cytokine Array

To profile the response of stimulated HT-29 epithelial cells, 38 cytokines were simultaneously analyzed using a Human cytokine array kit (Cat no. ARY005, R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Qualitative analysis

was performed by comparing the density of the detected spots to that of the reference spots included in the assay.

Stimulation of PBMCs by Purified Bacteriophages

Ethical Permission

Peripheral blood mononuclear cells (PBMCs) from healthy blood donors were obtained. The samples were treated according to the by-law of the ethical permission granted by the Karolinska Institute, Stockholm, Sweden (Permit no. 2014/2052-32).

PBMC Preparation

PBMC were isolated by Ficoll–Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient centrifugation and then cyro-preserved in liquid nitrogen. Prior to use, cells were thawed and washed three times in pre-warmed RPMI-1640 complete medium (Invitrogen, Sweden) supplemented with 10%(v/v) FCS, 1%(v/v) PEST, 1%(v/v) L-glutamine, and 2%(v/v) HEPES (HyClone Laboratories, Inc.) and checked for viability. PBMC (1×10^6 cells/mL) were then seeded into flat-bottomed 96-well plates (Sarstedt Inc.) and co-cultured with three different concentrations of four phages (10^9, 10^7, and 10^5 PFU/well) for 24 h for cytokine analysis and a single concentration of 10^9 PFU/well for 96 h for MTT assay at 37°C in a 5% CO_2 atmosphere. For cytokine analysis, negative (complete media only) and positive (50 ng/mL LPS) controls were also performed. For MTT assays, cells treated with water and dimethyl sulfoxide (DMSO) acted as positive controls for cell toxicity, complete medium was used as a negative control and a-CD3/a-CD28 beads (Invitrogen) acted as a proliferation control. Supernatants from PBMC stimulations were collected and frozen at −20°C until analyzed for cytokine content and the cells were used for MTT assay.

ELISA for Cytokine Stimulation of PBMC

ELISA was performed on purified phage preparations of different concentrations (10^9, 10^7, and 10^5 PFU/well) using commercially available kits IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α (Mabtech AB, Stockholm, Sweden) according to the manufacturer's instructions. Optical density (OD) was determined at 405 nm on a micro-plate reader (Molecular Devices Corp, Sunnyvale, CA, USA). Data were analyzed in the SoftMax Pro 5.2 rev C software package (Molecular Devices Corp, Sunnyvale, CA, USA).

Cell Proliferation and Toxicity Assay

Post-stimulation, 100 μL/well of their respective complete medium containing 9.1% 12 mM MTT solution (Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Sweden) was added into the PBMC and IEC (Caco-2 and HT-29) culture wells and the plates were incubated at 37°C for 3 h. MTT solution containing complete media was also added in an empty well as blank control. The supernatant was then removed, and the purple formazan crystals formed were solubilized with 50 μL/well of DMSO (Sigma-Aldrich, MO, USA). The absorbance was determined using an automatic microplate spectrophotometer at 540 nm. The OD values for each stimulus were adjusted for the contribution of the blank control (Peng et al., 2007).

Endotoxin Quantitation

Purified phage preparations and bacterial debris were checked once for endotoxin content using a Pierce Limulus amebocyte lysate (LAL) Chromogenic Endotoxin Quantitation Kit (Thermo Fisher, Sweden) according to the manufacturer's instructions.

Statistical Analysis

A one way ANOVA was used for the analysis of normally distributed whole data sets, 2-tailed *t*-tests for pairwise comparisons within normalized data sets, Kruskal–Wallis tests for non-normally distributed whole data sets or Mann–Whitney for pairwise comparisons between non-normalized data. Statistical analysis was performed in the R Statistical software package (R Core Team, 2015). Individual statistical tests used are identified in the figure legends or text where necessary.

RESULTS

Phages Maintain Lytic Activity in the Presence of Human Epithelial Cells

When incubated with epithelial cells and hosts, phages were able to significantly reduce the bacterial content over a period of 8 h (**Figure 1A**; $P < 0.0001$ by ANOVA). This reduction in bacterial content was shown to be the result of productive phage replication by an increase in phage titer over the same period

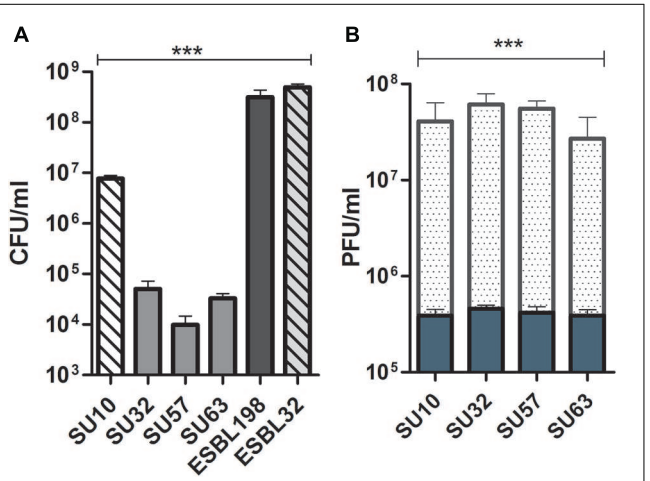

FIGURE 1 | Bactericidal activity and proliferation of phages in the presence of human epithelial cells. (A) Bactericidal efficacy of phages in the presence of epithelial cells. Efficacy is compared to bacteria only controls. Solid bars are ESBL198. Hatched bars are ESBL32. **(B)** Active replication of phages in the presence of host and epithelial cells; solid bars are phages and epithelial cells only, dotted bars are phages in the presence of host and epithelial cells. Reproduction in the presence of host is compared to phages exposed to epithelial cells. Results from HT-29 ($n = 3$) and Caco-2 ($n = 3$) cells were combined and data are presented as mean ± SD ***$P < 0.0001$ by one way ANOVA.

(**Figure 1B**; $P < 0.0001$ by ANOVA). It should also be noted that all phages, were reduced in number following 8 h incubation with epithelial cells when compared to the initial inoculum (from 5×10^5 to the lowest 3×10^5; $P < 0.05$ by two tailed *t*-test). For SU32 this reduction was smaller (from 5×10^5 to the lowest 4×10^5; $P > 0.05$ by two tailed *t*-test). No significant impact on the efficacy and stability of phages was observed between the two cell lines.

Phages Activate Pro-inflammatory Cytokines in HT-29 Cells

When phages were incubated with HT-29 cells in the absence of a suitable host, cytokines IL-8, CXCL-1/GROα and Macrophage migration inhibitory factor (MIF) were induced (**Table 1**). In the presence of a bacterial host (ESBL-198), a more intense pattern of cytokine activation was observed for phages SU32, SU57, and SU63. An additional cytokine, IL-1ra/IL-1F3 was activated in the presence of ESBL-198 only.

Due to the host range of SU10, a second ESBL containing *E. coli* (ESBL-32) was chosen for comparative analysis. This combination of phage and host induced an additional pair of cytokines, IL-18/IL-1F4 and IL-1ra/IL-1F3 in the presence of HT-29 cells (**Table 1**). These additional cytokines were also present in the ESBL-32 control.

Inflammatory and Anti-inflammatory Immune Response Varies between Phages

When assessed at the highest phage concentration (10^9 PFU/well) all samples were found to induce IL-6, IL-10, and TNF-α (**Figure 2**) at varying levels depending on the phage/cytokine combination. When compared to the medium (negative) control, IL-6 was significantly induced by all phages ($P < 0.002$ by Mann–Whitney) with the exception of SU32. Both IL-10 and TNF-α were significantly induced by SU57 and SU63 ($P < 0.05$ by Mann–Whitney) but not by SU10 or SU32 when compared to the negative control. When compared to the positive (LPS) control, all phages were significantly less immunostimulatory ($P < 0.05$ by Mann–Whitney) with the exception of SU63. TNF-α induction levels for SU57 were insignificant when compared to LPS. When comparing the ratio of TNF-α to IL-10, phage treated samples were higher than the medium control ($P > 0.05$ by Mann–Whitney). At lower phage concentrations (10^7 and 10^5 PFU/well) no significant induction of cytokines were observed (data not shown). IFN-γ was induced by all four phages but only in PBMCs obtained from two donors. The levels of IL-2, IL-4, and *IL-17* were below the limit of detection for all conditions tested. In addition, no response to the purified bacterial debris when incubated with PBMC was observed (data not shown).

Phages Are Non-toxic to Human Cells

In a long term assessment of the cytotoxic effects of individual phages on IECs and PBMCs, phages at a concentration of 10^9 PFU/well were not lethal to the HT-29 and Caco-2 cells after 96 h of incubation ($P < 0.05$ by two tailed *t*-test) and no significant differences between the cell lines were observed (**Figure 3**). Phage stimulated IECs produced a significantly ($P < 0.05$ by two tailed *t*-test) higher OD response compared to the negative control. Phages were unable to induce a significant cytotoxic effect on PBMCs.

LAL Analysis of Phage Preparations May Not Be Suitable for Quality Control Purposes

Phage preparations at the highest concentrations (10^9 PFU/mL) showed marked differences in endotoxin content compared to the bacterial debris control.

DISCUSSION

Phage therapy has enjoyed renewed interest for the treatment of multi-drug resistant bacteria due to increasing global levels of resistance. Despite this renewed interest, a number of concerns still exist over their safety and efficacy, which require substantial investigation. Depending on the route of administration, phages would require a level of persistence in the presence of, or the ability to evade, innate and adaptive immune components in order to reach a target site and produce a therapeutic

TABLE 1 | Cytokine release from HT-29 cells when exposed to four structurally distinct phages.

Cytokine	Bacteriophage				Bacteria and phage				Controls		
	SU10	SU32	SU57	SU63	ESBL-198			ESBL-32	ESBL-198	ESBL-32	Medium
					SU32	SU57	SU63	SU10			
IL-8	++	+	+	+	++	+++	+++	++	+++	+++	–
IL-18/IL-1F4	–	–	–	–	–	–	–	++	–	++	–
CXCL1/ GROα	++	+	+	+	++	++	+++	++	++	++	–
IL-1ra/IL-1F3	–	–	–	–	–	–	–	+++	++	+++	–
MIF	+	++	++	++	++	++	++	+++	+++	+++	++

+++*Pixel intensity ≥ reference spot.*
++*Pixel intensity < reference spot.*
+*Pixel intensity < than reference and test (bacteria and phage) spots.*
–*No response detected.*

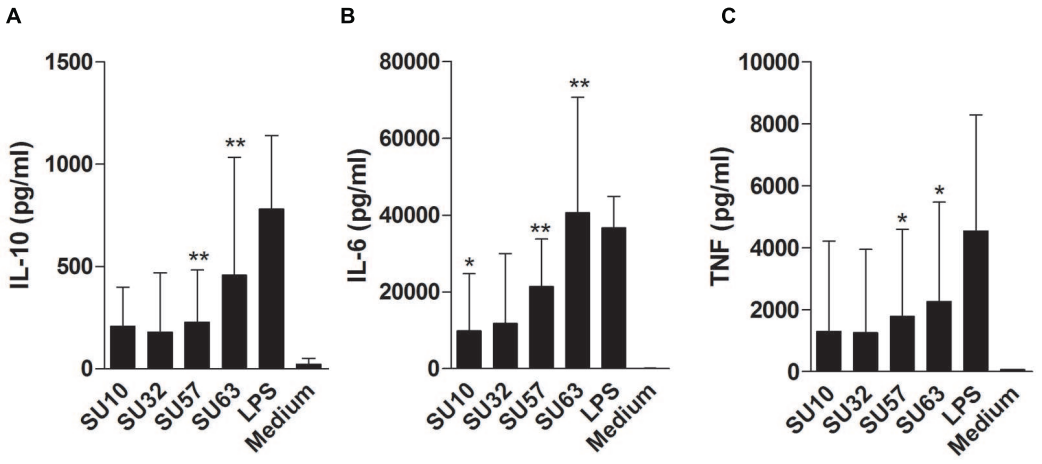

FIGURE 2 | Cytokine release by peripheral blood mononuclear cells (PBMCs) following exposure to high concentrations (10^9 PFU/well) of four different purified bacteriophages. (A) IL-10 release. **(B)** IL-6 release. **(C)** TNF-α release. Data are the mean of two replicates taken from six donors ± SD. $^{**}P < 0.001$; $^{*}P < 0.05$ by Mann–Whitney. Cytokine induction by phages is compared to medium.

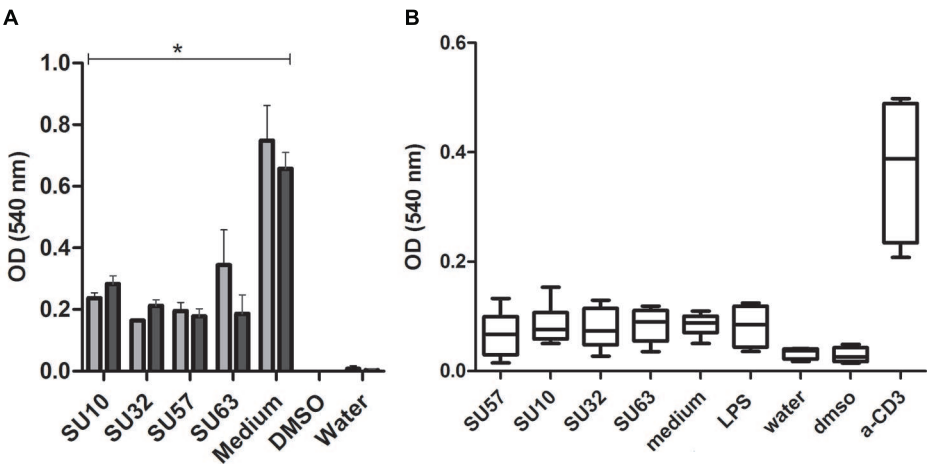

FIGURE 3 | Cell cytotoxicity and inhibition of proliferation by exposure to phages. (A) Phage exposure to intestinal epithelial cells; HT-29 ($n = 4$) ■ and Caco-2 ($n = 4$) ■. Data are presented as the mean ± SD. $^{*}P < 0.05$ by two tailed *t*-test compared to the medium control. **(B)** Phage exposure of PBMCs. Data are from two replicates from four donors.

effect (Abedon, 2011; Chan and Abedon, 2012). In the current study, all phages were shown to maintain killing efficacy and replicative ability in the presence of IECs and a suitable bacterial host. However, individual phages varied in their ability to elicit an immunogenic response with SU57 (Siphoviridae) and SU63 (Myoviridae) being the most immunogenic and SU32 (Siphoviridae) the least and suggests that phage immunogenicity cannot be generalized between families (e.g., SU57, SU63) or within the same family (e.g., SU32 and SU57). Despite the temptation to generalize the immunogenic properties of phages, recent studies have demonstrated that different proteins within the T4 phage possess different levels of immunogenicity (Dabrowska et al., 2014; Majewska et al., 2015). In order to ascertain the differences in immunogenicity between phage families (e.g., Myoviridae, Siphoviridae, or Podoviridae) or individual phages (e.g., SU63 and SU32 in the current work), comparisons between protein content and tertiary structures would be required.

Impact on Phage Efficacy and Viability When Exposed to Human Epithelial Cells

In order to be a viable adjuvant or alternative to conventional antibiotic therapy, whole phage therapeutics requires the establishment of a productive infection lifecycle. Following administration, this would require the migration of phages to the site of bacterial infection and the ability to persist in the presence of, or evade, the immune system while maintaining an infectious dose (Abedon and Thomas-Abedon, 2010; Abedon, 2011; Bull and Gill, 2014). ESBL-32 was chosen as a specific host for SU10

for the current investigation in order to produce comparable levels of activity between the four phages (Khan Mirzaei and Nilsson, 2015). Despite the maintenance of high killing efficacy and reproductive ability in the presence of IECs and suitable bacterial hosts (**Figure 1**), the number of phages reduced with a different level in the absence of bacterial hosts compared to the initial inoculum which could be due to difference in their stability under the experimental conditions. However, this low level of clearance would not explain the disparity between the activities of different phages, i.e., approximately 2–3 $\log_{10}$ difference between SU10 and the other phages and may result from the unique infection dynamics of each phage (Bull et al., 2014). Although not considered in this study, the *in situ* environmental conditions (e.g., gut pH) may potentially have a detrimental effect on the stability and efficacy of the phages.

Cytokine Release and Immune Stimulation Following Phage Exposure

The current study shows the varying degrees of immunogenicity exhibited by four distinct phages of the Caudovirales order when stimulating human PBMCs and IECs. IL-18 activation is of current interest as a vaccine adjuvant due to the ability to activate T-cells in the presence of different bacteria and cell types (Miettinen et al., 1998; Sugimoto et al., 2004). It is generally believed that phages are safe for human use, however, the lack of appropriate clinical trials and a lack of regulatory approval have restricted routine clinical use to specialist centers around the world (Skurnik and Strauch, 2006; Sarker et al., 2012). However, the current investigation suggests that both SU57 and SU63 at high concentrations (10^9 PFU/well) can significantly induce IL-6 and TNF-α from PBMCs compared to the negative (medium) control. SU57 and SU63 were also able to significantly induce the anti-inflammatory cytokine IL-10 compared to the negative (medium) control. In addition to this, SU10 also activates IL-6 release with a significant level from PBMCs compared to the negative (medium) control. Despite a lack of toxicity in both HT-29 and Caco-2 cells, phage exposure inhibited cell proliferation via an unknown mechanism although there is some suggestion that the interaction between phages and epithelial cells may result in the rapid release of reactive oxygen species (Gorski et al., 2012).

Endotoxin Determination in Phage Preparations

In order to satisfy regulatory requirements for non-topical clinical application, particularly intravenous injection, it is necessary to quantify endotoxin levels in the final product (Merabishvili et al., 2009). This is routinely accomplished with the "gold standard" method of an LAL assay. However, a number of studies suggest that the use of LAL assays results in ambiguous results when applied on phages (Merabishvili et al., 2009; Cooper et al., 2014) and was again highlighted in the current study. This discrepancy between LAL results and immune response studies may be the result of phages/LAL cross reactivity. Although supplemental methods, including *in vivo* assessments exist, these methods may not provide the required level of sensitivity necessary for pharmaceutical quality control and also raise ethical concerns over routine animal usage. An ELISA assay to profile immune responses to formulations may provide a sensitive alternative to LAL assessment and address ethical concerns over routine animal usage as has been suggested by Hodyra-Stefaniak et al. (2015) and should be the focus of future work.

CONCLUSION

The level of immunogenicity varies between the phages tested with SU57 and SU63 stimulating a greater release of IL-10, IL-6, and TNF-α compared to SU10 and SU32 in PBMCs. Despite this immunostimulatory activity, all phages were able to maintain high levels of lytic activity and establish reproductive cycles in the presence of suitable bacterial hosts and IECs. This may suggest that should sufficient numbers of phages reach a site of infection then a therapeutic effect may arise. All phages halted the growth rate of IECs compared to the control medium. The current investigation has highlighted the importance of immunological studies for the development of phages for use in human therapy and also that cell line studies may provide an additional method for endotoxin detection and quantification.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MKM, YH, ES-E, AN. Performed the experiments: MKM, YH, MN, CC. Analyzed the data: MKM, YH, CC, AN, ES-E. Wrote the manuscript: MKM, CC, YH, AN, ES-E.

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Commentary: Morphologically Distinct *Escherichia coli* Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release *In Vitro*

Nicolas Dufour[1, 2, 3], *Marine Henry*[3], *Jean-Damien Ricard*[1, 2, 4] *and Laurent Debarbieux*[3]*

[1] AP-HP, Hôpital Louis Mourier, Service de Réanimation Médico-Chirurgicale, Colombes, France, [2] INSERM, Infection Antimicrobials Modelling Evolution, UMR 1137, Paris, France, [3] Department of Microbiology, Molecular Biology of Gene in Extremophiles, Institut Pasteur, Paris, France, [4] Université Paris Diderot, Infection Antimicrobials Modelling Evolution, UMR 1137, Paris, France

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A commentary on

Morphologically Distinct *Escherichia coli* Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release *In Vitro*

by Khan Mirzaei, M., Haileselassie, Y., Navis, M., Cooper, C., Sverremark-Ekström, E., and Nilsson, A. S. (2016). Front. Microbiol. 7:437. doi: 10.3389/fmicb.2016.00437

In their recent paper, Khan Mirzaei et al. investigated the pro-inflammatory potential of bacteriophages (Khan Mirzaei et al., 2016). They addressed a crucial question linked to the safety of phage therapy, especially when the administration of bacteriophages is anticipated to be performed on highly reactive body compartments (e.g., the bloodstream by intravenous injection or the lung alveolar area by nebulization).

Using peripheral blood mononuclear cells from healthy donors they measured the release of three cytokines (TNF, IL-6, and IL-10) following incubation with four different bacteriophage solutions. As routinely performed, the positive control consisted in lipopolysaccharide extract (LPS) and the negative control in cell culture medium (low endotoxin controlled medium). When using the highest amount of bacteriophages (10^9 PFU/well), the authors observed that most of the solutions led to a significant increase in the acute pro-inflammatory cytokines (IL-6, TNF-α), compared to the level obtained with the negative control.

Surprisingly, the four bacteriophages solutions elicited a very high release of pro-inflammatory cytokines, with average values ranging from 0.25 to 1-fold the values obtained with the positive LPS control. In particular, bacteriophage SU63 was found to be as potent as LPS to induce an IL-6 secretion with a value as high as 40,000 pg/mL.

Such results raise the question of the quality of the bacteriophage preparations in terms of endotoxin level. Since a universally approved method for the preparation of bacteriophages for human application (or animals, including those used in experimental phage therapy models) is still lacking, we should make our best efforts to fully document the method used.

Here the authors followed a well-known protocol starting from polyethylene glycol precipitation of a bacterial lysate followed by a cesium chloride (CsCl) ultracentrifugation step and most likely a dialysis against a buffer which composition is not specified. No further purification step seems to have been undertaken before these solutions were tested for their pro-inflammatory potential. We believe and subsequently present supporting data that such a protocol is not sufficient to remove endotoxin contamination from CsCl-purified bacteriophage solutions.

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Peter Mullany, UCL, UK

Reviewed by:
Masaaki Murakami, Hokkaido University, Japan
Krystyna Dabrowska, Institute of Immunology and Experimental Therapy Wrocław, Poland

***Correspondence:**
Laurent Debarbieux
laurent.debarbieux@pasteur.fr

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In our laboratory, we use a different protocol based on concentration/washing by ultrafiltration and two CsCl ultracentrifugations (a step gradient followed by an isopycnic gradient) (Henry et al., 2013). After the dialysis step, we perform an affinity chromatography dedicated to endotoxin removal (EndoTrap Blue, Hyglos, Germany). This last step, repeated 3 to 5 times, is easily carried out using commercially available columns and can guarantee, in most cases, a very low level of endotoxin, usually below 0.5 EU/mL.

When we measured the endotoxin level present in solutions dialyzed following CsCl ultracentrifugations, we found them to be quite high (see **Figure 1** for 4 independent solutions). It is only after the third passage through the endotoxin removal column that low levels were reached. The use of such endotoxin removal methods applied to bacteriophage solutions were, to our knowledge, first reported in 2004 (Boratynski et al., 2004) and are considered as a required step when producing bacteriophage batches for clinical applications (Merabishvili et al., 2009) or immunological studies (Majewska et al., 2015). In the absence of such appropriate procedures, Cooper et al. (2014) have measured a gigantic level of endotoxin (>1,000,000 EU/mL) in a cocktail of bacteriophages prepared for nebulization and obtained from a crude lysate passed through a rudimentary 0.2 μm filter for sterilization.

Therefore, only purified bacteriophage solutions showing the lowest achievable endotoxin level (which may vary for each bacteriophage preparation), should be used to perform immunological tests. Otherwise, inaccurate conclusions could be made by attributing to bacteriophages an effect that originates from residual endotoxins (or other pro-inflammatory molecules). Moreover, apart from endotoxin, which triggers Toll-like receptor-4 (TLR-4), bacterial lysates may also contain several pathogen-associated molecular patterns (PAMPs) able to elicit a pro-inflammatory response, such as flagellin (sensed by TLR-5), unmethylated CpG Oligodeoxynucleotide DNA (TLR-9), lipoteichoic acid from Gram-positive bacteria (TLR-2) and triacyl lipopeptides (TLR-1 with TLR-2) (Akira and Hemmi, 2003).

We can agree with the concluding remarks from Khan Mirzaei et al., who suggest using ELISA assays to profile the immune response induced by phage formulations in order to provide a sensitive alternative to endotoxin assessment, but only if such a profiling method is formerly bound to an appropriate purification protocol dedicated to potent pro-

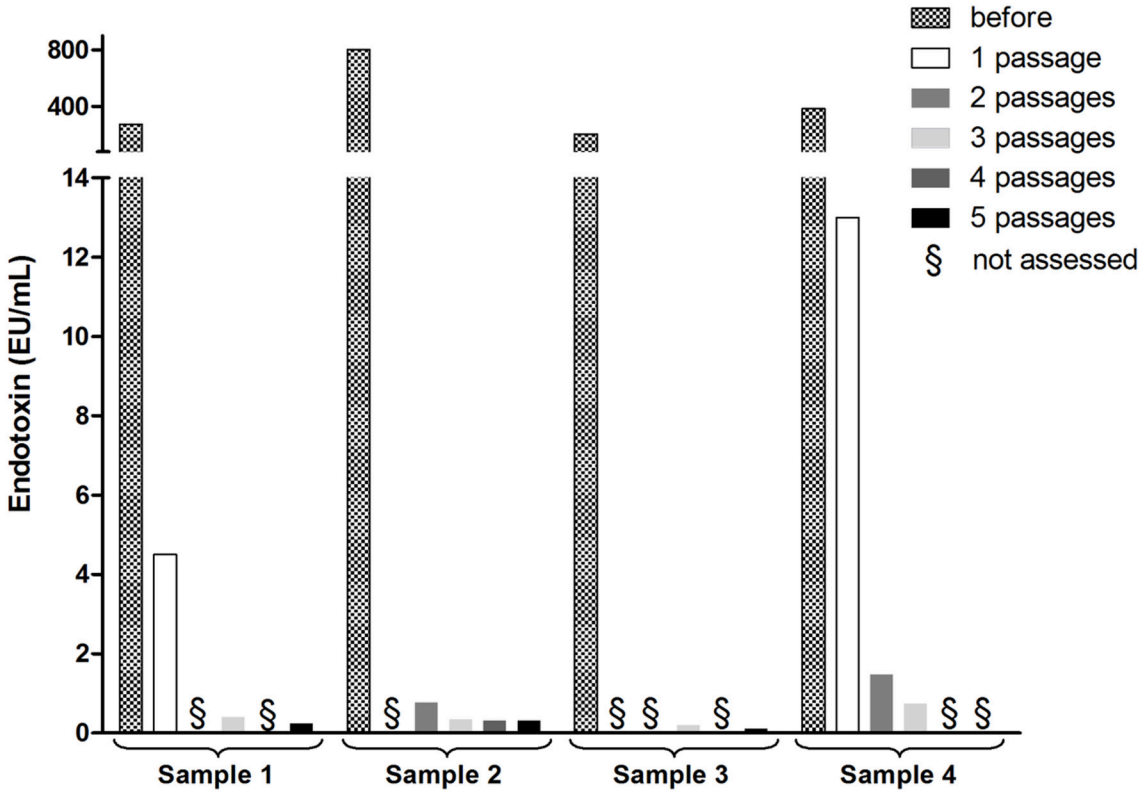

FIGURE 1 | Endotoxin levels in four independent preparations of bacteriophage PAK-P1 infecting *Pseudomonas aeruginosa* (Henry et al., 2013). Limulus amebocyte lysate-based assays (Endozyme rFC assay, Hyglos, Germany) were carried out before and after 1 to 5 passages of the solutions through a specific endotoxin removal column (Endotrap blue, Hyglos, Germany). Before the specific endotoxin removal step, each bacterial lysate (500 mL) was sterilized with two in-line filters (pore sizes, 0.8 to 0.45 and 0.2 to 0.1 μm; Sartopore 300; Sartorius) and concentrated/washed with an ultrafiltration cassette (Vivaflow 200; Sartorius). The concentrates were then ultracentrifuged twice on cesium chloride gradients and dialyzed against Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5). Final concentration ranged from 10^9 to 10^{11} pfu/mL. Similar results were observed with coliphages.

inflammatory molecules removal. Unfortunately for patients, when bacteria succumb to a viral attack, they do not release a ready-to-use pharmaceutical grade bacteriophage solution!

AUTHOR CONTRIBUTIONS

ND, JR, and LD wrote this letter, MH performed the experiment provided as example.

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Response: Commentary: Morphologically Distinct *Escherichia coli* Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release *In Vitro*

Mohammadali Khan Mirzaei[1], *Yeneneh Haileselassie*[2], *Marit Navis*[2], *Callum Cooper*[2], *Eva Sverremark-Ekström*[2] *and Anders S. Nilsson*[2]*

[1] Department of Microbiology and Immunology, McGill University, Montreal, QC, Canada, [2] Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

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Edited by:
Peter Mullany,
University College London, UK

Reviewed by:
António Gil Castro,
University of Minho, Portugal

***Correspondence:**
Anders S. Nilsson
anders.s.nilsson@su.se

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A commentary on

Commentary: Morphologically Distinct *Escherichia coli* Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release *In Vitro*
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In their recent commentary on our manuscript entitled "Morphologically Distinct *Escherichia coli* Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release *In vitro*," Dufour et al. suggest that the level of cytokine response generated in our paper is due to remaining bacterial debris rather than true differences between individual phages (Dufour et al., 2016).

Although at their core the two preparative methods are similar (both require filtration, CsCl centrifugation and dialysis). The additional purification step (the EndoTrap Blue system) binds endotoxins based on the conserved core of lipopolysaccharide (http://www.hyglos.de/en/products-services/products/endotoxin-removal/faq.html), which to our understanding is achieved through lipopolysaccharide specific binding to a phage derived protein. However, as Dufour et al. rightly point out, "bacterial lysates may also contain several pathogen-associated molecular patterns (PAMPs) able to elicit a pro-inflammatory response, such as flagellin (sensed by TLR-5), unmethylated CpG Oligodeoxynucleotide DNA (TLR-9), lipoteichoic acid from Gram-positive bacteria (TLR-2) and triacyl lipopeptides (TLR-1 with TLR-2) (Akira and Hemmi, 2003)." While such additional debris components may initially be in the minority of remaining debris, they would not be removed by the Endotrap system and may not be quantified as part of a Limulus amebocyte lystate (LAL) assay. Indeed, there have been previous instances where LAL assessment of phage preparations required alternative quantification methods (Merabishvili et al., 2009) or produced non-comparative results to an ELISA based system (Szermer-Olearnik and Boratyński, 2015). Therefore, had a true comparative analysis between the preparative methods and the same bacterial species been performed, differences could potentially have been less. However, to our knowledge no such studies have been performed.

With hindsight and a thorough data audit, we would certainly concur that at least some component of the observed cytokine responses are due to remaining contaminants and

that additional purification steps such as chromatography will further reduce possible contaminants. However, such additional steps could come at a cost, reduce overall phage concentration and potentially require additional concentration steps (Boratyński et al., 2004). As such we would suggest appropriate caution when interpreting our data and would highlight that the other data presented in the paper are consistent with the position that phages are not directly cytotoxic (Merabishvili et al., 2009; Chhibber et al., 2014; Henein et al., 2016).

However, while it is likely that some component of the cytokine response generated is due to remaining contaminants, additional variation could also be introduced through factors which are uncontrollable, such as genetic variation in Toll receptors between different donors (Netea et al., 2012).

Despite the suggestion to the contrary provided by the Dufour et al. commentary, the reduction and quantification of endotoxin in phage preparations remains an active area of research (Branston et al., 2015; Szermer-Olearnik and Boratyński, 2015) as more effective, less labor intensive, and perhaps most importantly for commercial development, more cost effective methods are sought. However, this highlights the disparity between preparative methods for phage assessment and once again shows the urgent need for standardized methods and acceptance criteria, similar to those employed in antibiotic and biocide development to be developed.

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Corrigendum: Morphologically Distinct *Escherichia coli* Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release *In Vitro*

*Mohammadali Khan Mirzaei †, Yeneneh Haileselassie, Marit Navis, Callum Cooper, Eva Sverremark-Ekström and Anders S. Nilsson **

Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

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Pilar García, Spanish National Research Council, Spain

****Correspondence:***
Anders S. Nilsson
anders.s.nilsson@su.se

†Present Address:
Mohammadali Khan Mirzaei, Department of Microbiology and Immunology, McGill University, Montreal, QC, Canada

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A corrigendum on

Morphologically Distinct *Escherichia coli* Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release *In Vitro*

by Khan Mirzaei, M., Haileselassie, Y., Navis, M., Cooper, C., Sverremark-Ekström. E., and Nilsson, A. S. (2016). Front. Microbiol. 7:437. doi: 10.3389/fmicb.2016.00437

In the original article, it was suggested that bacterial debris controls exhibited no cytokine response when incubated with PBMC. However, a subsequent data audit and additional statistical analysis has revealed that a number of the bacterial debris controls exhibited a positive cytokine response whereas others not, resulting in an inflated mean particularly for the TNF response (Supplementary Table 1). These means were not significantly different to the response generated by the purified phages. This does not impact on the data presented or the statistical analysis that has been performed as part of Figure 2 (analysis was compared to commercial LPS or medium only) or any of the other analysis performed as part of the manuscript. However, it does mean that the bacterial debris controls are not suitable for showing the efficacy of the phage purification process and as such a component of the cytokine response generated may be due to remaining bacterial debris as suggested by Dufour et al. (2016).

Therefore, the sentence "In addition, no response to the purified bacterial debris when incubated with PBMC was observed (data not shown)" (page 4, end of second paragraph) is inaccurate and should consequently not be considered when evaluating the effect of phage preparations on human cell lines as described in our article.

In addition, there is an error in the figure legend of Figure 3A. The colors used to differentiate between the HT-29 and Caco-2 cells have become inverted.

The authors apologize for these two mistakes. While the first does not impact the conclusions from the statistical analyses that have been performed, as the comparisons of immune response of phage preparations by PBMCs were made against a standardized LPS control and not against the purified bacterial debris controls, it does support the suggestion made by Dufour et al. (2016) that at least some component of the observed cytokine response generated by the phage preparations may be due to residual contaminants.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.02145/full#supplementary-material

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Dufour, N., Henry, M., Ricard, J. D., and Debarbieux, L. (2016). Commentary: morphologically distinct *Escherichia coli* bacteriophages differ in their efficacy and ability to stimulate cytokine release *in vitro*. *Front. Microbiol.* 7:1029. doi: 10.3389/fmicb.2016.01029

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ORIGINAL RESEARCH
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LPS-Activated Monocytes Are Unresponsive to T4 Phage and T4-Generated *Escherichia coli* Lysate

Katarzyna Bocian[1], Jan Borysowski[2], Michał Zarzycki[1], Piotr Wierzbicki[2], Danuta Kłosowska[2], Beata Weber-Dąbrowska[3], Grażyna Korczak-Kowalska[1,2] and Andrzej Górski[2,3]*

[1] Department of Immunology, Faculty of Biology, University of Warsaw, Warsaw, Poland, [2] Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland, [3] Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

A growing body of data shows that bacteriophages can interact with different kinds of immune cells. The objective of this study was to investigate whether T4 bacteriophage and T4-generated *Escherichia coli* lysate affect functions of monocytes, the key population of immune cells involved in antibacterial immunity. To that end, we evaluated how T4 and *E. coli* lysate influence the expression of main costimulatory molecules including CD40, CD80 and CD86, TLR2, TLR4 on monocytes, as well as the production of IL-6 and IL-12 in cultures of peripheral blood mononuclear cells (PBMCs). Separate experiments were performed on unactivated and LPS-activated PBMCs cultures. Both studied preparations significantly increased the percentage of $CD14^+CD16^-CD40^+$ and $CD14^+CD16^-CD80^+$ monocytes in unactivated PBMCs cultures, as well as the concentration of IL-6 and IL-12 in culture supernates. However, neither purified T4 nor *E. coli* lysate had any significant effect on monocytes in LPS-activated PBMCs cultures. We conclude that LPS-activated monocytes are unresponsive to phages and products of phage-induced lysis of bacteria. This study is highly relevant to phage therapy because it suggests that in patients with infections caused by Gram-negative bacteria the administration of phage preparations to patients and lysis of bacteria by phages are not likely to overly stimulate monocytes.

Keywords: bacteriophage, T4, phage therapy, bacterial lysate, *Escherichia coli*, monocyte

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Victor Krylov, I.I.Mechikov Institute for Vaccines & Sera, Russia

****Correspondence:***
Grażyna Korczak-Kowalska
korczak-kowalska@wp.pl

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INTRODUCTION

Bacteriophages are increasingly considered as a means of treatment of bacterial infections, including those caused by antibiotic-resistant bacteria (Międzybrodzki et al., 2012; Kutter et al., 2015). However, during treatment, phages can interact not only with bacteria, but also with different populations of immune cells including those involved in the induction of antibacterial immune responses (Górski et al., 2012). An important kind of immune cells engaged in antibacterial immunity are monocytes (Lauvau et al., 2015). These are cells of myeloid origin that constitute 5–12% of leukocytes in the peripheral blood in humans. The life span of monocytes in blood is up to 2 days. While unactivated monocytes undergo apoptosis, activation by different factors associated with ongoing infection or inflammation results in apoptosis inhibition, and monocytes migrate into inflamed tissues, phagocytose apoptotic cells, cellular debris, and different

particles. In addition, monocytes can differentiate into macrophages and inflammatory dendritic cells (iDCs; Swirski et al., 2014). Along with DCs and macrophages, monocytes comprise a heterogeneous population of mononuclear phagocytes (MPS) involved in antigen processing and presentation to initiate and regulate immune responses to pathogens, vaccines, tumors, and tolerance to autoantigens. Main functions of the MPS system include tissue maintenance and healing, innate immunity and pathogen clearance, and the induction of adaptive immune responses (Reynolds and Haniffa, 2015).

Monocytes are one of the key populations of immune cells to combat bacterial infections. Apart from direct antibacterial effects mediated by phagocytosis, reactive oxygen species, and nitric oxide, monocytes are also involved in regulation of both innate and adaptive immune responses during infections (Lauvau et al., 2015). Given a central role of monocytes during bacterial infections, we investigated whether bacteriophages could affect functions of this population of cells. Because during ongoing infection monocytes can be activated by different components of bacterial cells including lipopolysaccharide (LPS; Salomao et al., 2012), we also included to our study experiments to investigate whether bacteriophages affect functions of LPS-activated monocytes. Our study focused on $CD14^{+}CD16^{-}$ classic monocytes, the most abundant subpopulation of these cells (Ziegler-Heitbrock, 2015).

The study was performed on T4 phage that for decades has been the model phage in various studies and has been extensively characterized at the genetic and molecular level (Leiman et al., 2003). Moreover, T4 was used in previous studies of the effects of phages on the immune system (Górski et al., 2012), as well as in the first randomized placebo-controlled safety test of phage therapy (Bruttin and Brüssow, 2005).

MATERIALS AND METHODS

Bacteriophage

T4 phage was obtained from American Type Culture Collection (ATCC; USA) and was propagated on *E. coli* B from the Collection of Microorganisms of the L. Hirszfeld Institute of Immunology and Experimental Therapy (IIET), Wrocław, Poland.

Purified preparation of T4 phage was prepared by Laboratory of Bacteriophages, IIET, according to the protocol reported in detail by Boratyński et al. (2004). In brief, phage purification involved sequential ultrafiltration of crude T4 phage-generated *E. coli* B lysate through polysulfone membranes followed by chromatography on sepharose 4B (Sigma–Aldrich, Poland) and cellulofine sulfate (Millipore, USA). Stock preparations of T4 were suspended in phosphate-buffered saline (PBS; Biomed, Poland). Phage titer was measured by two-layer method of Adams (Adams, 1959).

The concentration of LPS in purified T4 phage preparation was determined using QLC-1000 Endpoint Chromogenic LAL test kit (Lonza, Switzerland) according to the manufacturer's instructions. The concentration of LPS in the preparation was 3 ng/ml. Therefore, in immunological experiments, LPS (Sigma–Aldrich, Poland) diluted with PBS was used at a concentration of 3 ng/ml as an additional control for purified T4 phage preparation.

Bacterial Lysate

T4 phage-generated bacterial lysate was prepared by Laboratory of Bacteriophages, IIET, according to the protocol reported by Slopek et al. (1983) and Letkiewicz et al. (2009). In brief, T4 was incubated with *E. coli* B in LB medium (Sigma–Aldrich, USA) at 37°C until complete bacterial lysis (approx. 4–6 h). Next the suspension was filtered through a 0.22-μm filter (Millipore, USA). Stock preparation of the lysate was suspended in peptone water (IIET, Poland). Phage titer in lysate was measured by two-layer method of Adams (Adams, 1959).

In immunological experiments an additional control for T4-generated *E. coli* lysate was peptone water.

Cell Cultures

All experiments were performed on cells isolated from healthy blood donors. Informed, written consent was obtained from all donors. The study protocol was approved by the ethics committee of the Medical University of Warsaw. Peripheral blood mononuclear cells (PBMCs) were isolated from blood specimens by density-gradient centrifugation over Gradisol L (Aqua Medica, Poland). PBMCs were cultured at a density of 1×10^6/ml in RPMI medium (Biomed, Poland) supplemented with FCS (Sigma–Aldrich, USA), L-glutamine (Sigma–Aldrich, USA), HEPES (Sigma–Aldrich, USA), and gentamicin (Krka, Slovenia) in 24-well plates at 37°C with 5% CO_2 for 24 h. In each experiment, two parallel cultures were set up. In one culture, PBMCs were activated with LPS (Sigma–Aldrich; 10 μg/ml), and in the other cells were treated with equal volume of PBS. Simultaneously, in some cultures purified T4 phage (10^8 PFU/ml; final concentration), *E. coli* lysate (containing T4 phage at the final concentration of 10^8 PFU/ml), control LPS (3 ng/ml), or peptone water was also added to wells. In control cultures equal volume of PBS was added to wells. After 24 h of culture viability of PBMCs was determined using trypan blue. The viability of cells was consistently 95%. PBMCs were harvested for flow cytometry analysis of surface markers, and culture supernates were frozen at −20°C for measurements of cytokines concentrations.

Evaluation of Expression of Monocytes Surface Markers

Cells were incubated with the following monoclonal antibodies: CD14-PerCP (BD Pharmingen, USA), CD16-FITC (BD Pharmingen), CD80-FITC (BD Pharmingen), CD86-PE (BD Pharmingen), CD40-PE (BD Pharmingen), TLR2-FITC (eBioscience, USA), and TLR4-PE (eBioscience). In isotype controls cells were stained with IgG1 conjugated with the respective fluorochromes (BD Pharmingen, eBioscience). After 30 min of incubation at 4°C, cells were washed twice in FACS buffer (Becton Dickinson, USA). The expression of monocytes surface markers was measured by flow cytometry (FACSCalibur, Becton Dickinson) and analyzed by Cell Quest software (Becton

Dickinson). The results were based on analysis of at least 100,000 cells and were shown as the percentage of positively labeled cells. Moreover, the mean fluorescence intensity (MFI) values of gated monocytes positive for individual markers were determined.

Determination of Cytokine Production

The concentrations of IL-6 and IL-12 (p70) were measured in cell culture supernates by enzyme-linked immunoassay (ELISA) using Human IL-6 and IL-12 p70 ELISA Ready-SET-Go kits (eBioscience) according to the manufacturer's instructions.

Statistics

Statistical analysis of the results was performed by Wilcoxon's matched pairs test. $P < 0.05$ was considered significant.

RESULTS

In order to investigate the effects of T4 bacteriophage and T4-generated *E. coli* lysate on monocytes, we determined: (1) the expression of main costimulatory molecules including CD40, CD80, and CD86, (2) the expression of TLR2 and TLR4 molecules, and (3) the production of IL-6 and IL-12. In each case, separate experiments were performed on unactivated and LPS-activated PBMCs.

In the first set of experiments, we evaluated whether T4 bacteriophage and T4-generated *E. coli* lysate affect the expression of CD40, CD80, and CD86 in $CD14^+CD16^-$ monocytes. In unactivated PBMCs cultured with T4 phage, the mean percentage of $CD14^+CD16^-CD40^+$ monocytes was 45.47 ± 19.5, while in the control (unactivated PBMCs to which equal volume of PBS was added) this percentage was 16.55 ± 7.61 ($p = 0.016$; **Figure 1A**). However, a comparable increase in the mean percentage of $CD14^+CD16^-CD40^+$ cells was observed in unactivated PBMCs cultured with control LPS (58.99 ± 25.39); the difference between the mean percentage of $CD14^+CD16^-CD40^+$ monocytes in cultures to which T4 phage or control LPS was added was not statistically significant. Similarly to T4 phage, T4-generated *E. coli* lysate significantly increased the mean percentage of $CD14^+CD16^-CD40^+$ monocytes to 52.81 ± 17.02 ($p = 0.008$ compared with the control). On the other hand, we found no significant differences between the MFI values of CD40 in $CD14^+CD16^-$ monocytes from PBMCs cultures to which PBS, T4 phage, control LPS, or *E. coli* lysate was added (**Table 1**). In LPS-activated PBMCs cultures, the percentage of $CD14^+CD16^-CD40^+$ monocytes was comparable in all culture variants (65.6 ± 14.17, 65.49 ± 17.1, 63.49 ± 15.87, and 59.37 ± 37 for variants to which PBS, T4 phage, control LPS, or *E. coli* lysate was added, respectively). Furthermore, in LPS-activated PBMCs cultures the MFI values of CD40 in $CD14^+CD16^-$ monocytes were also comparable in all culture variants (**Table 1**).

Moreover, we found that T4 phage significantly increased the mean percentage of $CD14^+CD16^-CD80^+$ monocytes in unactivated PBMCs cultures compared with the control (77.55 ± 13.31 and 37.91 ± 14.06 for cells cultured in the presence of T4 and control cells, respectively; $p = 0.0006$; **Figure 1B**); however, a similar increase in the mean percentage of $CD14^+CD16^-CD80^+$ monocytes was observed in cultures to which control LPS was added (80.61 ± 11.38). Like T4 phage, *E. coli* lysate significantly increased the mean percentage of $CD14^+CD16^-CD80^+$ monocytes (71.2 ± 14.75; $p = 0.0019$ compared with the control). In addition, we found that in unactivated PBMCs cultures, T4, control LPS, and *E. coli* lysate significantly increased the MFI values of CD80 in $CD14^+CD16^-$ monocytes compared with the control ($p = 0.0063$, $p = 0.00238$, and $p = 0.0379$ for T4 phage, control LPS, and *E. coli* lysate, respectively; **Table 1**).

In LPS-activated PBMCs, the mean percentage of $CD14^+CD16^-CD80^+$ monocytes was comparable in cultures to which PBS, T4 phage, control LPS, or *E. coli* lysate was added (95.61 ± 3.16, 96.41 ± 3.55, 87.26 ± 12.38, and 95.17 ± 3.13, respectively; **Figure 1B**). Furthermore, in LPS-activated PBMCs, we found no statistically significant differences between the MFI values of CD80 in $CD14^+CD16^-$ monocytes from individual culture variants (**Table 1**).

We also found that neither T4 phage nor *E. coli* lysate had any significant effect on the mean percentage value of $CD14^+CD16^-CD86^+$ monocytes in unactivated PBMCs; these values were 90.8 ± 11.3, 83.3 ± 11.31, and 73.56 ± 13.25 in cultures to which PBS, T4 phage, or lysate was added (**Figure 1C**). The corresponding MFI values were also comparable in all culture variants (**Table 1**). Likewise, in LPS-activated PBMCs, we found no significant differences between the mean percentage of $CD14^+CD16^-CD86^+$ monocytes in individual culture variants (73.56 ± 13.25, 90.87 ± 8.0, and 94.33 ± 6.87 in cultures to which PBS, T4 phage, or *E. coli* lysate was added, respectively; **Figure 1C**). In addition, we found that in LPS-activated PBMCs neither of the studied preparations significantly affected the MFI value of CD86 in $CD14^+CD16^-$ monocytes (**Table 1**).

In the second set of experiments, we evaluated whether T4 phage and *E. coli* lysate affect the expression of TLR2 and TLR4 on $CD14^+CD16^-$ monocytes. Overall, neither of the studied preparations significantly affected the expression of TLR2 or TLR4. In unactivated PBMCs the mean percentage of $CD14^+CD16^-TLR2^+$ cells was comparable in all culture variants (75.81 ± 15.87, 90.42 ± 6.8, and 83.54 ± 9.21 in cultures to which PBS, T4 phage, or lysate was added; **Figure 2**). In LPS-activated PBMCs the corresponding values were 83.21 ± 25.21, 88.69 ± 8.8, and 87.98 ± 12.71. The mean percentage of $CD14^+CD16^-TLR4^+$ cells in unactivated PBMCs cultures was 51.53 ± 35.74, 47.73 ± 39.01, and 60.02 ± 34.02 in control cultures and cultures to which T4 phage or lysate was added (**Figure 2**). In LPS-activated PBMCs these values were 65.04 ± 38.4, 47.73 ± 39.01, and 60.02 ± 34.02.

Moreover, we evaluated whether the investigated preparations stimulate the production of pro-inflammatory cytokines IL-6 and IL-12. We found that both T4 phage and *E. coli* lysate significantly increased the concentration of IL-6 in supernates of unactivated PBMCs cultures compared with the control (the mean concentration of IL-6 3.47 ± 2.62, 111.58 ± 15.11, and 99.68 ± 5,32 pg/ml in control cultures and cultures to which T4 or lysate was added, respectively; $p = 0.0294$ for both T4 and

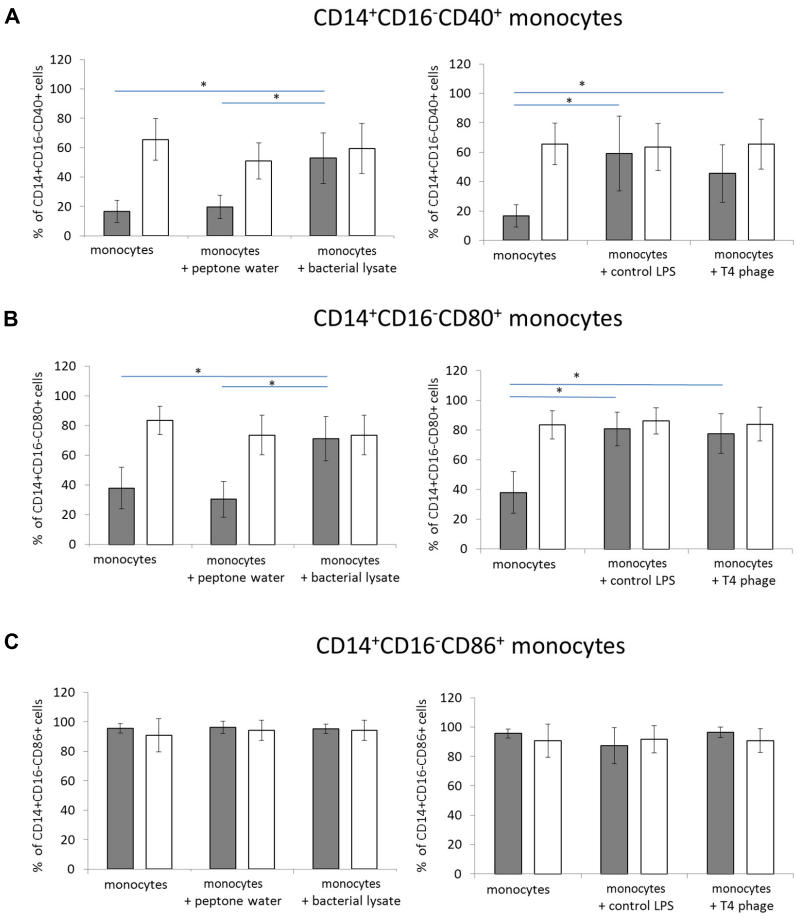

FIGURE 1 | The effects of T4 phage and T4-generated *Escherichia coli* lysate on the expression of costimulatory molecules on monocytes. The percentage values of CD40$^+$ **(A)**, CD80$^+$ **(B)**, and CD86$^+$ **(C)** cells were determined by flow cytometry in CD14$^+$CD16$^-$ monocytes from 24-h cultures of peripheral blood mononuclear cells (PBMCs). The expression of each marker was determined separately on monocytes from cultures of unactivated (gray bars) and LPS-activated (white bars) PBMCs. T4 phage or *E. coli* lysate was present in culture medium throughout the culture. Shown are the mean percentage values ± standard deviation. Statistically significant differences ($p < 0.05$) are marked with *.

lysate; **Figure 3**). A comparable increase in IL-6 concentration was observed in supernates of unactivated PBMCs cultures to which control LPS was added (115.99 ± 10.86 pg/ml; $p = 0.0294$ compared with the control; **Figure 3**). In LPS-activated PBMCs cultures the mean IL-6 concentration exceeded 100 pg/ml and was comparable in all culture variants (**Figure 3**).

Similar results were obtained for IL-12. Both T4 and *E. coli* lysate significantly increased the concentration of this cytokine in supernates of unactivated PBMCs cultures compared with the control (mean IL-12 concentrations 0.0, 8.22 ± 4.25, 5.42 ± 2.25 pg/ml in control cultures and cultures to which T4 or lysate was added, respectively; $p = 0.211$ for both T4 and lysate; **Figure 3**). IL-12 concentration was also significantly increased in supernates of unactivated PBMCs cultured with control LPS (19.92 ± 8.75 pg/ml; $p = 0.0211$ compared with the control; **Figure 3**). In supernates of LPS-activated PBMCs cultures the concentration of IL-12 was above 10 pg/ml and, we found no significant differences between individual culture variants (**Figure 3**).

DISCUSSION

The objective of this study was to evaluate whether bacteriophages can affect functions of monocytes, a key population of immune cells involved in antibacterial immunity (Lauvau et al., 2015). We focused on CD14$^+$CD16$^-$ classic monocytes, the most abundant subpopulation of monocytes (Ziegler-Heitbrock, 2015). All experiments were performed on T4 phage, a model dsDNA bacterial virus that has been extensively characterized at the genetic and molecular level (Leiman et al., 2003). Apart from purified preparation of T4, we used T4-generated *E. coli* lysate. *In vitro* experiments involving use of lysate were intended to mimic the effects of lysis of bacteria in a patient treated with a phage preparation. Thus, by using lysate, we investigated whether monocytes functions can be affected by products of phage-induced lysis of bacterial cells. Moreover, lysates are used in the treatment of bacterial infections at some centers of phage therapy (Międzybrodzki et al., 2012).

TABLE 1 | The mean fluorescence intensity (MFI) of costimulatory molecules and TLRs on monocytes cultured with T4 phage and T4-generated *Escherichia coli* lysate.

		CD40	CD80	CD86	TLR2	TLR4
Monocytes	Unactivated	28.75 (± 8.44)	59.05 (± 11.82)	374.04 (± 318.90)	64.72 (± 23.77)	62.61 (± 22.95)
	Activated LPS 10 ug/ml	37.62 (± 21.42)	150.56 (± 118.72)	153.66 (± 66.54)	125.37 (± 66.10)	63.11 (± 26.05)
Peptone water +	Monocytes unactivated	29.20 (± 5.46)	59.53 (± 19.55)	399.41 (± 315.84)	71.97 (± 24.78)	47.43 (± 15.37)
	Monocytes activated LPS 10 ug/ml	29.65 (± 12.38)	96.21 (± 49.61)	148.48 (± 81.07)	104.11 (± 47.23)	65.04 (± 27.26)
Control LPS +	Monocytes unactivated	44.55 (± 16.76)	*131.06 (± 87.81)	150.05 (± 66.72)	94.09 (± 60.03)	50.01 (± 22.77)
	Monocytes activated LPS 10 ug/ml	38.94 (± 20.31)	150.34 (± 105.49)	160.18 (± 81.20)	151.84 (± 69.18)	55.67 (± 19.85)
T4 phage +	Monocytes unactivated	34.49 (± 11.65)	*134.02 (± 74.33)	275.61 (± 128.34)	103.92 (± 57.42)	49.42 (± 15.61)
	Monocytes activated LPS 10 ug/ml	41.04 (± 26.74)	147.55 (± 103.47)	164.68 (± 76.70)	111.88 (± 44.85)	87.26 (± 86.90)
Bacterial lysate +	Monocytes unactivated	36.94 (± 11.90)	*114.75 (± 61.45)	194.53 (± 90.40)	116.61 (± 58.27)	87.44 (± 41.24)
	Monocytes activated LPS 10 ug/ml	34.46 (± 18.15)	107.77 (± 63.2)	181.53 (± 70.96)	130.88 (± 62.69)	52.43 (± 15.36)

*The MFI of costimulatory molecules (CD40, CD80, CD86), TLR2, and TLR4 was determined by flow cytometry in $CD14^+CD16^-$ monocytes from 24-h cultures of peripheral blood mononuclear cells (PBMCs). All experiments were performed on unactivated PBMCs (results in white boxes) and LPS-activated PBMCs (results in gray boxes). T4 phage or E. coli lysate was present in culture medium throughout the culture. Shown are the MFI values ± standard deviation. Statistically significant differences ($p < 0.05$) are marked with *.*

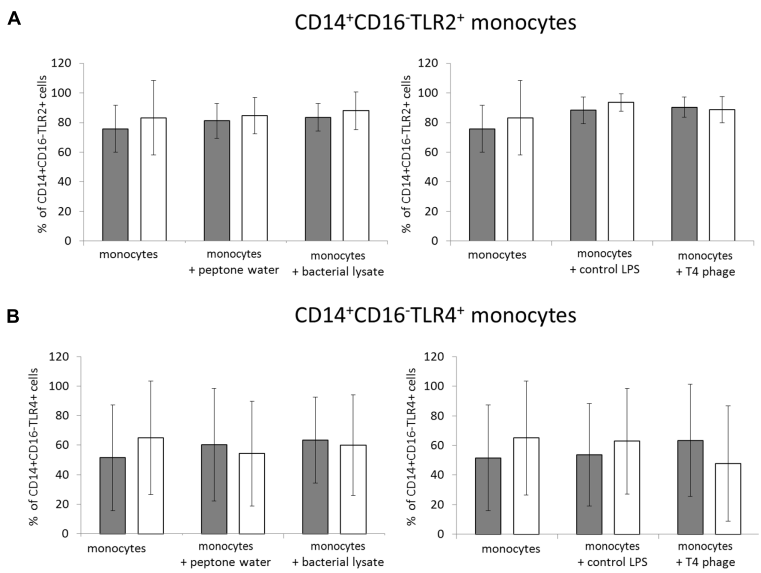

FIGURE 2 | T4 phage and T4-generated *Escherichia coli* lysate do not affect the expression TLR2 and TLR4 on $CD14^+CD16^-$ monocytes. The percentage values of $TLR2^+$ **(A)** and $TLR4^+$ **(B)** cells were determined by flow cytometry in $CD14^+CD16^-$ monocytes from 24-h cultures of PBMCs. The expression of both markers was determined separately on monocytes from cultures of unactivated (gray bars) and LPS-activated (white bars) PBMCs. T4 phage or *E. coli* lysate was present in culture medium throughout the culture. Shown are the mean percentage values ± standard deviation.

All experiments were performed on both unactivated and LPS-activated PBMCs. LPS is the principal component of Gram-negative bacterial cells known to activate monocytes in different infections including sepsis (Salomao et al., 2012). Experimental variants involving LPS-activated monocytes were included to evaluate whether bacteriophages can affect functions of monocytes that are in a state corresponding to that found in patients with ongoing bacterial infection. Recent microarray-based gene expression studies confirmed that in patients with bacterial infections, especially sepsis, PBMCs are activated, as

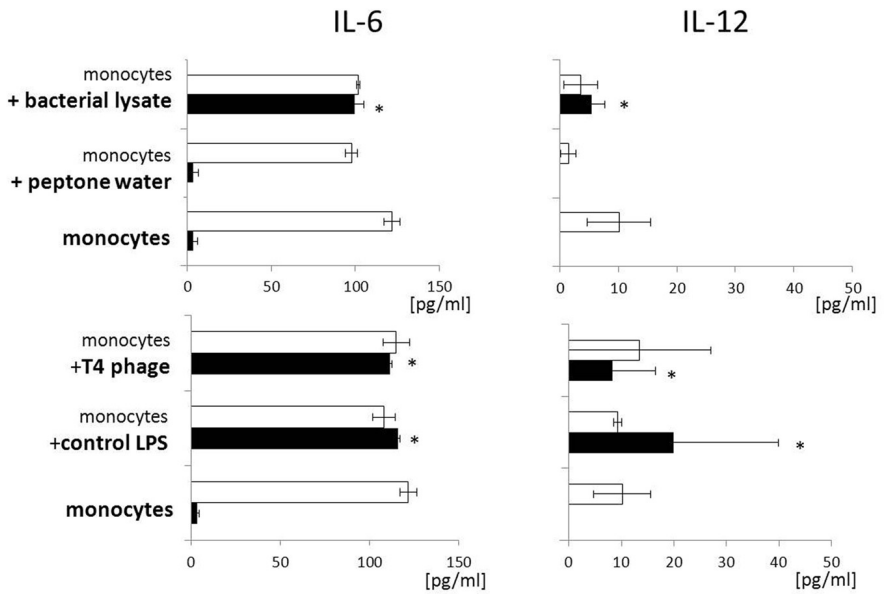

FIGURE 3 | T4 phage and T4-generated *Escherichia coli* lysate have no effect on the production of pro-inflammatory cytokines by LPS-activated PBMCs. Concentration of pro-inflammatory cytokines – IL-6 **(Left)** and IL-12 **(Right)** – was measured by ELISA in supernates from 24-h cultures of PBMCs. Production of both cytokines was evaluated separately in cultures of unactivated (black bars) and LPS-activated (white bars) PBMCs. T4 phage or *E. coli* lysate was present in culture medium throughout the culture. Shown are the mean concentrations ± standard deviation. Statistically significant differences ($p < 0.05$) are marked with *.

reflected by up-regulation of many genes involved in antibacterial immunity and inflammatory responses (Maslove and Wong, 2014; Severino et al., 2014).

We found that both purified T4 and *E. coli* lysate significantly increased the percentage of $CD14^+CD16^-CD40^+$ and $CD14^+CD16^-CD80^+$ monocytes in unactivated PBMCs cultures without any significant effect on the percentage of $CD14^+CD16^-CD86^+$ monocytes. However, we also observed a significant increase in the percentage of both $CD14^+CD16^-CD40^+$ and $CD14^+CD16^-CD80^+$ monocytes in unactivated PBMCs cultured with control LPS (used at a concentration of 3 ng/ml that corresponds to that found in T4 phage preparation). These findings suggest that the increase in the percentage of $CD14^+CD16^-CD40^+$ and $CD14^+CD16^-CD80^+$ monocytes observed in unactivated PBMCs cultured with purified T4 preparation was caused by residual LPS rather than bacteriophage virions themselves.

CD40, a member of the TNF receptor family, plays an important role in both cellular and humoral immune responses. Signaling through CD40 up-regulates the expression of a number of molecules including MHC class II, CD80, CD86, adhesion molecules and induces the production of cytokines such as IL-1, IL-6, IL-10, and TNF-α (Wu et al., 2009). Thus, our results suggest that in unactivated monocytes some products of phage-induced lysis of bacterial cells, especially LPS, may promote CD40-mediated humoral and cellular immune responses.

CD80 is a molecule whose expression on monocytes is increased following stimulation by some factors including LPS. CD80 can prolong or enhance costimulatory signals transmitted from antigen-presenting cells (APCs) to $CD4^+$ T cells thus promoting the activation of these cells (Rutkowski et al., 2003). Therefore, our data indicate that LPS, and possibly some other components of *E. coli* cells released from bacteria during phage-induced lysis can promote CD80-mediated costimulation of $CD4^+$ T cells.

Importantly, we also found that in LPS-activated PBMCs cultures the percentage of CD40, CD80, and CD86 on $CD14^+CD16^-$ monocytes was unaffected by purified T4 and *E. coli* lysate. These results suggest that in patients with infections caused by Gram-negative bacteria the administration of phage preparations, as well as lysis of bacteria by phage, is not likely to affect CD40-, CD80-, and CD86-mediated functions of monocytes.

We also showed that neither of the studied preparations significantly affected the expression of TLR2 and TLR4 on $CD14^+CD16^-$ monocytes both in unactivated and LPS-activated PBMCs cultures. TLRs are the key class of pattern-recognition receptors involved in the induction of innate immune responses to various pathogens (De Nardo, 2015). Thus our results suggest that neither phage nor products of lysis of bacterial cells are likely to affect TLR2- and TLR4-mediated immune responses.

Moreover, we showed that both T4 and *E. coli* lysate significantly increased the concentration of IL-6 and IL-12 in supernates of unactivated PBMCs cultures. While IL-6 and IL-12 are generally classified as "proinflammatory," in fact their actual biological effects are highly context-dependent and both cytokines may even exert anti-inflammatory activity (Chang and Radbruch, 2007; Hunter and Jones, 2015). Our previous observations indicate that lysates used for therapeutic purposes do not induce any proinflammatory effects in patients. For

example, lysates did not increase leukocytosis, sedimentation rate, and the concentration of C-reactive protein (CRP) in patients; furthermore, in individuals with the initial CRP concentration above 10 mg/l, a significant reduction in the concentration of this protein was shown between days 9 and 32 of treatment (Międzybrodzki et al., 2012). In line with our previous findings are the results of the present study that show that neither T4 nor *E. coli* lysate significantly increased the production of IL-6 and IL-12 in LPS-activated PBMCs cultures.

An important question is whether our results obtained for T4 phage and *E. coli* lysate can be extrapolated to other phages and bacterial lysates. At the present state of research it is hard to formulate general conclusions especially with respect to phages because it is known that structure of tailed phages is highly diversified and substantial differences in amino-acid sequences of phage proteins, including surface capsid proteins have been reported (Fokine and Rossmann, 2014). Therefore, it is possible that some phages have proteins that can activate some functions of monocytes. With respect to bacterial lysates, we believe that some differences between the effects of Gram-negative and Gram-positive bacteria might be expected due to different structure and composition of their cell walls (Silhavy et al., 2010). Thus, further studies should be conducted to evaluate whether activated monocytes are also refractory to other phages and phage-generated bacterial lysates.

CONCLUSION

While LPS, and possibly some other products of phage-induced lysis of *E. coli* affect the phenotype of unactivated monocytes and production of cytokines by unactivated PBMCs, LPS-activated cells are unresponsive to both phage virions and products of phage-induced lysis of bacterial cells. Our results are of importance to phage therapy because they suggest that the administration of phage preparations to patients with infections caused by Gram-negative bacteria and subsequent lysis of bacteria by phages are not likely to overly stimulate monocytes.

AUTHOR CONTRIBUTIONS

KB performed the experiments, analyzed data, and contributed to drafting of the manuscript. JB analyzed data and contributed to drafting of the manuscript. MZ, PW, and DK performed the experiments. BW-D analyzed data. GK-K conceived the study, analyzed data and contributed to drafting of the manuscript. AG conceived the study and contributed to drafting of the manuscript. All authors approved the final version of the manuscript.

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The Effects of T4 and A3/R Bacteriophages on Differentiation of Human Myeloid Dendritic Cells

Katarzyna Bocian[1], Jan Borysowski[2], Michał Zarzycki[1], Magdalena Pacek[2], Beata Weber-Dąbrowska[3], Maja Machcińska[1], Grażyna Korczak-Kowalska[1,2]* and Andrzej Górski[2,3]*

[1] Department of Immunology, Faculty of Biology, University of Warsaw, Warsaw, Poland, [2] Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland, [3] Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Bacteriophages (phages) are viruses of bacteria. Here we evaluated the effects of T4 and A3/R bacteriophages, as well as phage-generated bacterial lysates, on differentiation of human myeloid dendritic cells (DCs) from monocytes. Neither of the phages significantly reduced the expression of markers associated with differentiation of DCs and their role in the activation of T cells (CD40, CD80, CD83, CD86, CD1c, CD11c, MHC II, PD-L1, PD-L2, TLR2, TLR4, and CCR7) and phagocytosis receptors (CD64 and DEC-205). By contrast, bacterial lysate of T4 phage significantly decreased the percentages of DEC-205- and CD1c-positive cells. The percentage of DEC-205-positive cells was also significantly reduced in DCs differentiated in the presence of lysate of A3/R phage. Thus while bacteriophages do not substantially affect differentiation of DCs, some products of phage-induced lysis of bacterial cells may influence the differentiation and potentially also some functions of DCs. Our results have important implications for phage therapy of bacterial infections because during infections monocytes recruited to the site of inflammation are an important source of inflammatory DCs.

Keywords: bacteriophage, T4, A3/R, phage therapy, dendritic cell, differentiation, lysate

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****Correspondence:***
Grażyna Korczak-Kowalska
korczak-kowalska@wp.pl
Jan Borysowski
jborysowski@interia.pl

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INTRODUCTION

Bacteriophages (phages) are viruses of bacteria. Phages constitute an extremely abundant and diversified group of viruses. Thus far, over 6000 bacteriophages have been identified by using electron microscopy and the total number of phage virions in the biosphere has been estimated at around 10^{32}. Phages occur in great numbers in different environments including water, soil, and air. Moreover they are an important component of the microflora in humans and different animals (Letarov and Kulikov, 2009; Hatfull and Hendrix, 2011; Ackermann and Prangishvili, 2012; Dalmasso et al., 2014). The main biomedical application of bacteriophages is the treatment of bacterial infections; many studies performed on animal models of infections, observational clinical studies, and the first small randomized controlled trials of phage therapy indicate high safety and efficacy of phages as antibacterial agents (Kutter et al., 2014; Wittebole et al., 2014). In addition, a growing body of data shows that bacteriophages can affect functions of different populations of immune cells involved in both innate and adaptive immunity including neutrophils, monocytes, macrophages, as well as T and B cells (Borysowski et al., 2010; Górski et al., 2012).

An important population of immune cells critically involved in the regulation of immune responses are dendritic cells (DCs). DCs present antigens to lymphocytes, priming naïve T cells and driving them toward a specific lineage fate. The maturation state of DCs can be manipulated to boost or suppress immune responses. Several DC subpopulations have been identified based on typical surface molecule expression patterns. Each DC subset possesses unique functional specialization. The major population of human myeloid DCs (mDCs) in blood, tissues and lymphoid organs are $CD1c^+$ mDCs. They were originally identified in blood as a fraction of $HLA\text{-}DR^+$ lineage cells expressing myeloid antigens CD11b, CD11c, CD13, CD33, CD172a (SIRP α) and CD45RO. Human tissue $CD1c^+$ DCs tend to be more activated than their blood counterparts in terms of expression of CD80, CD83, CD86 and CD40. They have lost expression of homing receptors CLA and CD62L but up-regulated CCR7. $CD1c^+$ DCs are equipped with a wide range of lectins, Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) involved in antigen uptake, transport and presentation (Collin et al., 2013; McGovern et al., 2015; Poltorak and Schraml, 2015; Vu Manh et al., 2015).

Given the central role of DCs in the regulation of immune responses, research on the interactions between phages and DCs is essential for understanding the effects of bacterial viruses on the immune system. Over recent years the first studies have been conducted to investigate the influence of bacteriophages on some functions of DCs (Pajtasz-Piasecka et al., 2008; Sartorius et al., 2011; Miernikiewicz et al., 2013; An et al., 2014). All these studies were performed on murine bone marrow-derived dendritic cells (BMDCs); to the best of our knowledge, there are no data on the interactions between phages and human DCs. The results of studies of murine DCs cannot be simply extrapolated to human DCs because of substantial differences between functions of murine and human DCs; these differences pertain to the production of some cytokines, the expression of some surface molecules including TLRs, and the effects of viral stimulation (Robbins et al., 2008; Schlitzer et al., 2015). Therefore, in this study we asked whether bacteriophages can affect human DCs.

The main objective of this study was to evaluate the effects of two bacteriophages – T4 (specific for *Escherichia coli*) and A3/R (specific for *Staphylococcus aureus*) on the differentiation of human mDCs from monocytes *in vitro*. Moreover, we investigated whether mDCs differentiation can be affected by the products of phage-induced lysis of host bacteria.

MATERIALS AND METHODS

Bacteriophages

T4 phage was purchased from American Type Culture Collection (ATCC; USA) and was cultured on *E. coli* B obtained from the Collection of Microorganisms of the L. Hirszfeld Institute of Immunology and Experimental Therapy (IIET), Wrocław, Poland.

A3/R was obtained from the Phage Collection of IIET. It is a descendant of A3 phage which was originally obtained from Gerhard Pulverer, Institute of Hygiene, University of Cologne, Germany in 1986, and adapted to therapeutic use by several rounds of passaging through clinical *S. aureus* strains. A3/R was cultured on *S. aureus* strain 19930 from Laboratory of Bacteriophages, IIET.

Purified preparation of T4 phage was prepared by Laboratory of Bacteriophages, IIET, as described in detail elsewhere (Boratyński et al., 2004). In brief, the protocol involves sequential ultrafiltration of crude phage-generated *E. coli* lysate through polysulfone membranes followed by chromatography on sepharose 4B (Sigma-Aldrich, Poland) and cellulofine sulfate (Millipore, USA). To obtain purified preparation of A/3R, crude phage-generated *S. aureus* lysate was subjected to ultrafiltration through polysulfone membranes and chromatography on sepharose 4B. Stock phage preparations were suspended in phosphate-buffered saline (PBS). Phage titer was measured by two-layer method of Adams (1959). The concentration of LPS in purified phage preparations was determined using QLC-1000 Endpoint Chromogenic LAL test kit (Lonza) according to the manufacturer's instructions. In the A3/R preparation the concentration of LPS was undetectable, while in the T4 phage preparation it was 3 ng/ml. Therefore, as an additional control for T4 phage, LPS (Sigma-Aldrich, Poland) was used at a concentration of 3 ng/ml. LPS was diluted with PBS.

Bacterial Lysates

Phage-generated bacterial lysates were prepared by Laboratory of Bacteriophages, IIET, according to the modified method by Slopek et al. (1983; Letkiewicz et al., 2009). In brief, phages were incubated with their host bacteria in LB medium at 37°C until complete bacterial lysis occurred (approx. 4–6 h). Subsequently the suspension was filtered through a 0.22-μm filter (Millipore, USA). Stock preparations of both lysates were suspended in peptone water (IIET, Poland). Phage titer in lysates was measured by two-layer method of Adams (1959).

As an additional control in experiments to evaluate the effects of lysates on differentiation of DCs peptone water was used.

Generation of Myeloid Dendritic Cells from Peripheral Blood Monocytes

The study was performed on cells isolated from healthy blood donors. Informed, written consent was obtained from all donors and the study was approved by the ethical committee of the Medical University of Warsaw. Peripheral blood mononuclear cells (PBMCs) were isolated from blood specimens by density-gradient centrifugation over Gradisol L (Aqua Medica, Łódź, Poland). Isolation of monocytes from PBMCs was performed using Dynabeads® FlowComp™ Human CD14 (Invitrogen) according to the manufacturer's instructions. The purity of monocyte population was evaluated by flow cytometry (FACSCalibur, Becton Dickinson) using anti-CD14 monoclonal antibody (BD Pharmingen). The purity of the isolated monocytes was 98%. Monocytes were cultured at a density of 1×10^6/ml in the AIM-V medium (Gibco) supplemented with IL-4 (100 ng/ml; R&D Systems) and GM-CSF (250 ng/ml; R&D Systems) in 24-well plates at 37°C with 5% CO_2 for 5 days. In some cultures purified phage preparations or bacterial lysates were

added on day 0 to wells at a density of 10^8 PFU/ml (final concentration). In control cultures, equal volumes of PBS were added to wells. Differentiation of monocytes into mDCs was evaluated by flow cytometry (FACSCalibur, Becton Dickinson) using the following monoclonal antibodies: anti-CD11c-APC (BD Pharmingen), anti-CD1c-FITC (eBioscience), anti-CCR7-APC-e Fluor (eBioscience). In addition, cells were stained with anti-CD14-PerCP (BD Pharmingen) to confirm the state of differentiation of DCs which lose CD14 during differentiation. We consistently found that the percentage of CD14 cells in cultures was 98%. In addition, cell morphology during DCs differentiation was analyzed using ECLIPSE TE200 inverted microscope (Nikon) with a digital camera and the NIS-Elements software. On the last day of culture viability of DCs was determined using trypan blue. The viability of cells was 95%. Cultured cells were photographed and the expression of the investigated markers was determined by flow cytometry.

Evaluation of Expression of mDCs Surface Markers

Cells were incubated with appropriate monoclonal antibodies: CD11c-APCw (BD Pharmingen), CD1c-FITC (eBioscience), CD83-PE (BD Pharmingen), CD80-FITC (BD Pharmingen), CD86-PE (BD Pharmingen), CD40-PE (BD Pharmingen), PD-L1-FITC (BD Pharmingen), PD-L2-PE (BD Pharmingen), HLA-DR-PE (BD Pharmingen), CCR7-APC-e Fluor (eBioscience), TLR2-FITC (eBioscience), TLR4-PE (eBioscience), CD64-FITC (BD Pharmingen), and DEC-205-PE (BD Pharmingen). In addition, CD14-PerCP (BD Pharmingen) monoclonal antibody was used to confirm the differentiation of DCs. Isotype controls were cells stained with IgG1 conjugated with the respective fluorochromes (BD Pharmingen, eBioscience). After 30 min of incubation at 4°C, cells were washed twice in FACS buffer (Becton Dickinson). The expression of individual markers on gated $CD1c^+$ DCs was determined by flow cytometry (FACSCalibur, Becton Dickinson) and analyzed by Cell Quest software (Becton Dickinson). The results were based on analysis of at least 100,000 cells and were shown as the percentage of positively labeled cells. In addition, the mean channel fluorescence values of gated DCs positive for individual markers were determined.

Determination of Cytokine Production

The concentration of IL-12 (p70) was measured in cell culture supernatants by enzyme-linked immunoassay (ELISA) using Human IL-12 p70 ELISA Ready-SET-Go kit according to the manufacturer's instructions (eBioscience).

Statistics

Statistical analysis was performed by Wilcoxon's matched pairs test. $P < 0.05$ was regarded as significant.

RESULTS

To evaluate the effects of T4 and A3/R phages on the differentiation of mDCs, we analyzed the expression of main DC markers on mDCs differentiated from monocytes in the presence of individual preparations. We focused on two classes of markers: (i) molecules associated with maturation of DCs and their role in the activation of T cells (CD40, CD80, CD83, CD86, CD1c, CD11c, MHC class II, PD-L1, PD-L2, TLR2, TLR4, and CCR7), and (ii) receptors involved in phagocytosis (CD64 and DEC-205). In each case, we determined both the percentage of mDCs expressing a given marker and the value of the mean channel fluorescence of gated mDCs positive for this marker.

In general, both T4 and A3/R phage had no effect on the expression of any of the investigated markers. The percentages of cells expressing individual markers associated with differentiation of DCs and their role in the activation of T cells were comparable in mDCs generated in the presence of bacteriophages and in control cultures (**Figure 1**). Of note, we found some interpersonal variability in the level of CD80 expression compared with the level of expression of other analyzed markers.

Likewise, bacteriophages had no effect on the percentages of CD64- and DEC-205-positive mDCs (**Figure 2**). In addition, we found no significant differences between the expression of the analyzed markers on cells cultured in the presence of LPS (an additional control for T4 phage) and cells from control cultures (treated with PBS; data not shown).

In addition, we found no significant differences in the mean channel fluorescence values for the analyzed markers between mDCs differentiated in the presence of phages and in control cultures (**Table 1**).

Moreover, we did not observe any visible changes in morphology of mDCs generated in cultures to which phages were added compared with control cultures; in particular, we found no reduction in the number of cells with long dendrites, a hallmark of typical DC morphology (**Figure 3**).

Unlike purified bacteriophage preparations, phage-generated bacterial lysates affected the expression of some of the investigated markers. First, the percentages of DEC-205- and CD1c-positive cells were significantly reduced in mDCs differentiated in the presence of lysate of *E. coli* compared with mDCs from control cultures ($p = 0.001$ and $p = 0.002$, respectively; **Figures 1**, **2**, and **4**). Moreover, the percentages of TLR4- and PD-L2-positive mDCs were significantly higher in cultures to which the lysate was added compared with control cultures ($p = 0.012$ and $p = 0.018$, respectively; **Figures 1** and **4**); however, no significant difference was observed in the percentages of TLR4- and PD-L2-positive cells between mDCs generated in the presence of the lysate compared with mDCs differentiated in the presence of peptone water (data not shown).

We also found that in mDCs differentiated in the presence of lysate of *S. aureus* the percentage of DEC-205-positive cells was significantly reduced compared with mDCs from control cultures ($p = 0.016$; **Figures 2** and **4**). However, neither of the investigated lysates significantly affected the mean channel fluorescence values for individual markers (**Table 1**).

Representative density plots for markers for which statistically significant differences were found between mDC differentiated in the presence of lysates and mDCs from control cultures are shown in **Figure 4**.

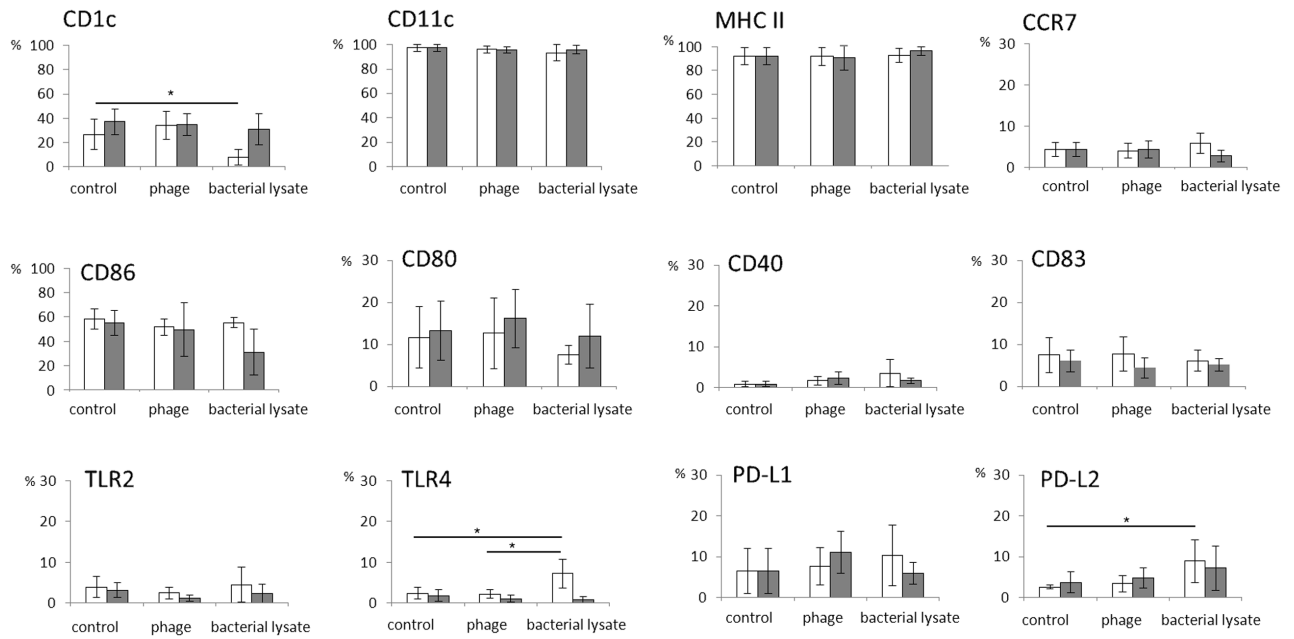

FIGURE 1 | The effects of bacteriophages and bacteriophage-generated bacterial lysates on the expression of markers associated with the differentiation of myeloid dendritic cells and their role in T cell activation. Human myeloid dendritic cells (mDCs) were differentiated from monocytes during five-day cultures with IL-4 (100 ng/ml) and GM-CSF (250 ng/ml) in the presence of individual phage preparations including purified preparation of T4 (white bars, ☐), T4-generated *Escherichia coli* lysate (white bars, ☐), purified preparation of A3/R (gray bars, ■), or A3/R-generated *Staphylococcus aureus* lysate (gray bars, ■). In control cultures cells were treated with equal volumes of PBS. The expression of all markers including CD40, CD80, CD83, CD86, CD1c, CD11c, MHC class II, PD-L1, PD-L2, TLR2, TLR4, and CCR7 was determined by flow cytometry. The bars show the percentages of mDCs expressing individual markers. The experiment was repeated five times. *$p < 0.05$.

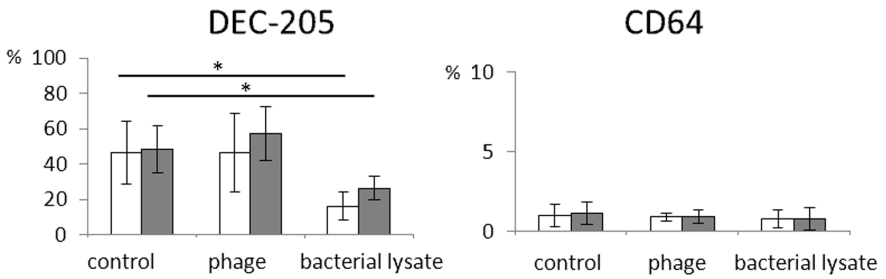

FIGURE 2 | The influence of bacteriophages and bacteriophage-generated bacterial lysates on the expression on myeloid dendritic cells of receptors involved in phagocytosis. Human mDCs were differentiated from monocytes during five-day cultures with IL-4 (100 ng/ml) and GM-CSF (250 ng/ml) in the presence of individual phage preparations including purified preparation of T4 (white bars, ☐), T4-generated *Escherichia coli* lysate (white bars, ☐), purified preparation of A3/R (gray bars, ■), or A3/R-generated *Staphylococcus aureus* lysate (gray bars, ■). In control cultures cells were treated with equal volumes of PBS. The expression on mDCs of DEC-205 and CD64 molecules was determined by flow cytometry. The bars show the percentages of mDCs expressing DEC-205 and CD64. The experiment was repeated five times. *$p < 0.05$.

We also observed that both lysates significantly reduced the number of cells with typical DC morphology (long dendrites) compared to control cultures (**Figure 3**).

Apart from the expression of main DC markers, we also investigated the production by mDCs of IL-12 p70. However, the level of IL-12 was undetectable in culture supernatants of mDCs generated in the presence of both purified phage preparations and lysates of *E. coli* and *S. aureus*, as well as in control cultures (data not shown).

DISCUSSION

The overall objective of this study was to evaluate the effects of T4 and A3/R bacteriophages on the differentiation of human mDCs from monocytes. In *in vivo* settings phages may affect function of immune cells not only by direct interactions, but also indirectly, by different components of bacterial cells released from lysed bacteria. Therefore, our study included experiments to investigate both direct effects of bacteriophage particles on mDCs and the

TABLE 1 | Geo mean values of surface markers of mDCs differentiated in the presence of bacteriophages and bacteriophage-generated bacterial lysates.

	Control		phage		bacterial lysate	
Geo Mean	**T4**	**A3/R**	**T4**	**A3/R**	***Escherichia coli* (T4)**	***Staphylococcus aureus* (A3/R)**
CD1c	23.2 (± 4.3)	24.7 (± 9.5)	23.6 (± 4.2)	24.8 (± 7.7)	20.6 (± 3.5)	22.8 (± 4.7)
CD11c	182.5 (± 67.3)	134.4 (± 36.3)	140.5 (± 38.5)	119.7 (± 32.4)	111.3 (± 23.3)	137.6 (± 35.4)
MHCII	77.2 (± 34)	50.2 (± 17.5)	70 (± 9.5)	47.6(18.5)	60.2 (± 14.9)	47.3 (± 8.1)
CCR7	36.3 (± 3.2)	46.4 (± 22.8)	35.8 (± 14.1)	58.8 (± 30.7)	50.3 (± 31.7)	43.8 (± 20.3)
CD86	62.5 (± 14.1)	63.8 (± 12.5)	65 (± 10.4)	67 (± 13.4)	57.8 (± 4.3)	57 (± 12.9)
CD80	51.6 (± 5.4)	57.8 (± 9.1)	47.8 (± 5.1)	60 (± 5.5)	59.2 (± 4.4)	44.2 (± 7)
CD40	29 (± 2.3)	28.7 (± 2.3)	25.6 (± 4.2)	26.4 (± 2)	25.6 (± 4.5)	28 (± 0.8)
CD83	18.4 (± 1.1)	20.7 (± 5.6)	18.2 (± 1.8)	20.5 (± 6.3)	18.4 (± 1.1)	21 (± 6.4)
TLR2	16.2 (± 1.8)	16.2 (± 1.6)	19.2 (± 3)	18.4 (± 3.6)	21.4 (± 6)	17 (± 1.2)
TLR4	29.3 (± 2.3)	28.4 (± 2.3)	33.7 (± 7)	29.2 (± 4.8)	31.8 (± 5.7)	33.8 (± 6.7)
PD-L1	25 (± 4.5)	23 (± 3.4)	22.6 (± 2.1)	23.4 (± 2.9)	29.4 (± 4.7)	29 (± 3.6)
PD-L2	17.4 (± 4.6)	17.4 (± 3.4)	17.4 (± 4.9)	18.6 (± 5.2)	21.8 (± 4.6)	18.8 (± 5.1)
DEC-205	42.8 (± 15.6)	42.8 (± 11.2)	41.2 (± 9.8)	46.4 (± 10.8)	53 (± 18.7)	36 (± 2.4)
CD64	29.8 (± 7)	29.9 (± 5.7)	24.7 (± 6.7)	22.6 (± 2.6)	22.8 (± 6.4)	23.3 (± 3.4)

influence of phage-generated bacterial lysates. Such lysates are also used in the treatment of bacterial infections at some centers of phage therapy (Międzybrodzki et al., 2012).

As model phages we employed T4 and A3/R. T4 is a classic phage that has been extensively characterized at the genetic and molecular level (Leiman et al., 2003). It belongs to the T4-like viruses genus of the *Tevenvirinae* subfamily of *Myoviridae* (Adriaenssens et al., 2012). Its dsDNA genome is 168 kb long and contains 278 open reading frames (ORFs) (Miller et al., 2003; Petrov et al., 2010). A3/R is a novel bacteriophage that was described recently (Łobocka et al., 2012). It is a strictly lytic, polyvalent staphylococcal phage from the Twort-like genus of the *Spounavirinae* subfamily of myoviruses. The dsDNA genome of A3/R is 132 kb long and contains 196 ORFs (GenBank Acc. No. JX080301).

We found that both the percentages of cells expressing individual markers and the mean channel fluorescence values for these markers were similar in mDCs generated in the presence of phages and in control cultures. These results indicate that T4 and A3/R do not adversely affect the differentiation of mDCs and the expression of molecules essential for some of DCs' main functions, especially a role in T cell activation and phagocytosis. The data obtained for T4 bacteriophage are in line with the results of Miernikiewicz et al. (2013) who showed that this phage does not significantly affect the expression of CD40, CD80, CD86, and MHC class II molecules on murine BMDCs *in vitro*. Likewise, in the study by Pajtasz-Piasecka et al. (2008), incubation with T4 did not substantially influence the expression of BMDC maturation markers including CD80, CD86, CD40, CD54, as well as class I and class II MHC. Potential effects on DCs of the other of the investigated bacteriophages – A3/R – have not been evaluated previously. In other studies, *Cronobacter sakazakii* ES2 phage was found to increase the expression of CD40, CD86, and MHC class II molecules on BMDCs (An et al., 2014), while filamentous bacteriophage fd did not affect the expression of CD40, CD80, CD86, MHC class I and MHC class II molecules on these cells (Sartorius et al., 2011). Thus individual phages can exert different effects on DCs.

It needs to be stressed that all previous studies on the interactions between bacteriophages and DCs have been conducted on BMDCs and their results cannot be simply extrapolated to human DCs. This results from the fact that there are substantial differences between murine and human DCs (Robbins et al., 2008; Dutertre et al., 2014; Schlitzer et al., 2015). To the best of our knowledge, our study is the first to evaluate the effects of phages on human DCs. In addition, a panel of DC markers analyzed in our study is considerably wider than the panels used in previous studies. For example, our study is the first to examine the effects of phages on the expression of some molecules associated with differentiation of DCs and their role in the activation of T cells (CD1c, CD11c, PD-L1, PD-L2, TLR2, TLR4, and CCR7), as well as of phagocytosis receptors (CD64 and DEC-205).

Recently, a specific subpopulation of DCs, called inflammatory DCs (infDCs) was described (Serbina et al., 2009; Segura and Amigorena, 2013; Dutertre et al., 2014; Schlitzer et al., 2015). InfDCs are generated from monocytes especially during inflammatory reactions (including those accompanying infections). The differentiation of monocytes into infDCs occurs at sites of ongoing inflammation. Thus, a finding that phages do not adversely affect the differentiation of monocytes into mDCs *in vitro* suggests that bacterial viruses should not inhibit the generation of infDCs *in vivo*; however, this notion needs to be verified by further studies.

It is known that immature DCs do not produce IL-12; the production of this cytokine is a feature of antigen-activated mature DCs (Albrecht et al., 2008; Turnquist et al., 2010). Thus, to evaluate whether bacteriophages can activate immature DCs, we determined the production of IL-12 by mDCs generated in the presence of T4 and A3/R. However, the level of

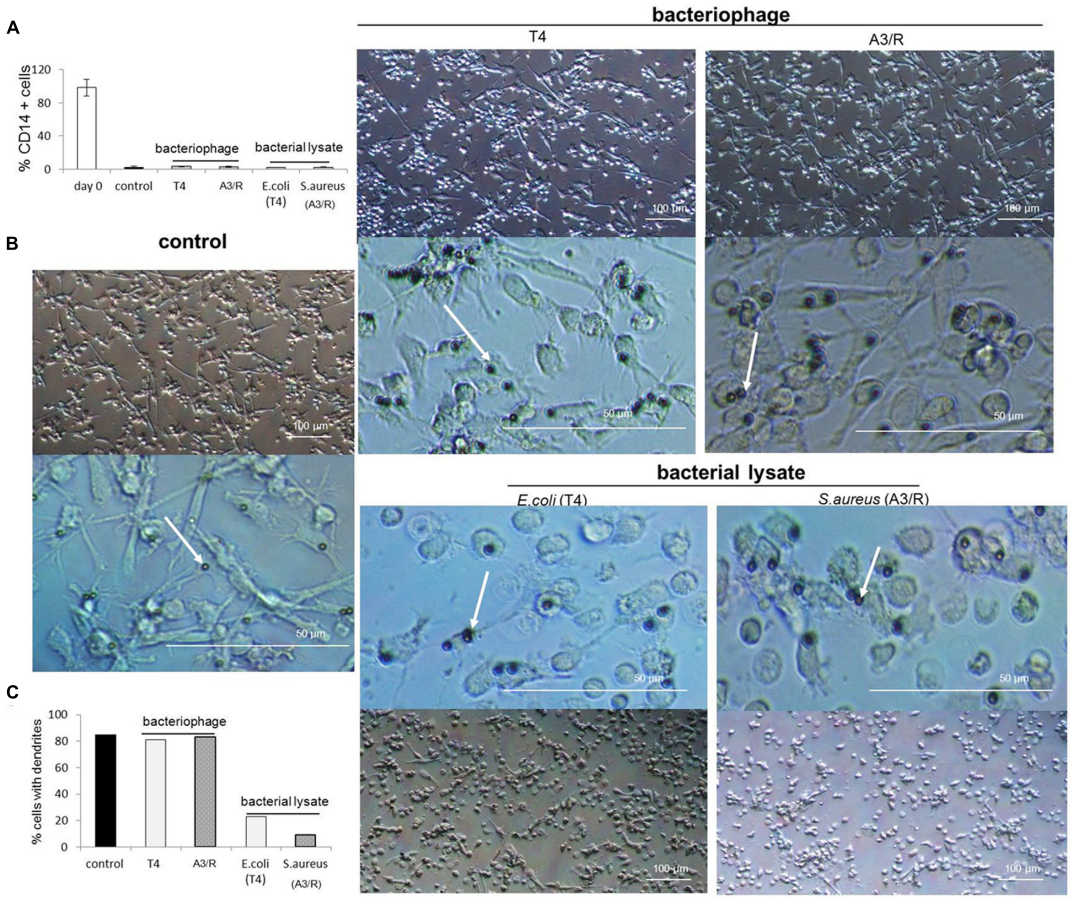

FIGURE 3 | Morphology of myeloid dendritic cells differentiated in the presence of bacteriophages and bacteriophage-generated bacterial lysates. Human mDCs were differentiated from $CD14^+$ monocytes during five-day cultures with IL-4 (100 ng/ml) and GM-CSF (250 ng/ml). The investigated preparations (T4 or A3/R bacteriophage, or the corresponding bacterial lysate) were added to wells on Day 0 of culture. In control cultures cells were treated with equal volumes of PBS. In all culture variants cells have lost the expression of CD14 during differentiation **(A)**. Morphology of mDCs differentiated in the presence of individual preparations is shown at lower and higher resolution. White arrows indicate microbeads attached to cell surface **(B)**. The presence of bacterial lysate of both T4 and A3/R phage resulted in a reduction in the number of dendrites in differentiated mDCs compared with mDCs from control cultures and mDCs generated in the presence of purified phage preparations **(B,C)**.

IL-12 was undetectable in culture supernatants from mDCs generated in the presence of bacteriophages, as well as in control cultures. This confirms that phages do not activate immature mDCs.

To evaluate whether the products of phage-induced lysis of bacterial cells can affect DCs, we used unpurified *E. coli* and *S. aureus* lysates generated *in vitro* as a result of infection of bacteria by T4 and A3/R, respectively. Bacterial lysates appear to be suitable model preparations for studying the effects of products of phage-induced lysis of bacterial cells generated *in vivo*. Moreover, investigating the effects of lysates is of practical importance because such preparations are used at some centers of phage therapy in the treatment of bacterial infections (Międzybrodzki et al., 2012). We found that bacterial lysate of T4 phage significantly decreased the percentages of DEC-205- and CD1c-positive mDCs. The percentage of DEC-205-positive cells was also significantly reduced in mDCs generated in the presence of lysate of *S. aureus*, while the expression of all other markers was unaffected. Remarkably, neither of the studied lysates substantially affected the mean channel fluorescence value for any marker. Furthermore, neither of the lysates stimulated the production by mDCs of IL-12.

DEC-205 (CD205) is a type I C-type lectin-like receptor involved in antigen uptake, processing, and presentation. Antigens endocytosed via DEC-205 were shown to enter both MHC class I and MHC class II antigen presenting pathways and to be consequently presented to both $CD4^+$ and $CD8^+$ T cells. Recently DEC-205 was found to recognize ligands expressed during apoptosis and necrosis by different cell populations, which confirms a role of this receptor in the induction of both central and peripheral tolerance to self-antigens (Shrimpton et al., 2009). CD1c belongs to Group 1 CD molecules. Its main function is the presentation of lipid antigens to different subpopulations of T cells. Lipids presented by CD1c include sphingomyelin, phosphatidylcholine, phosphatidylinositol, sulfatides, and

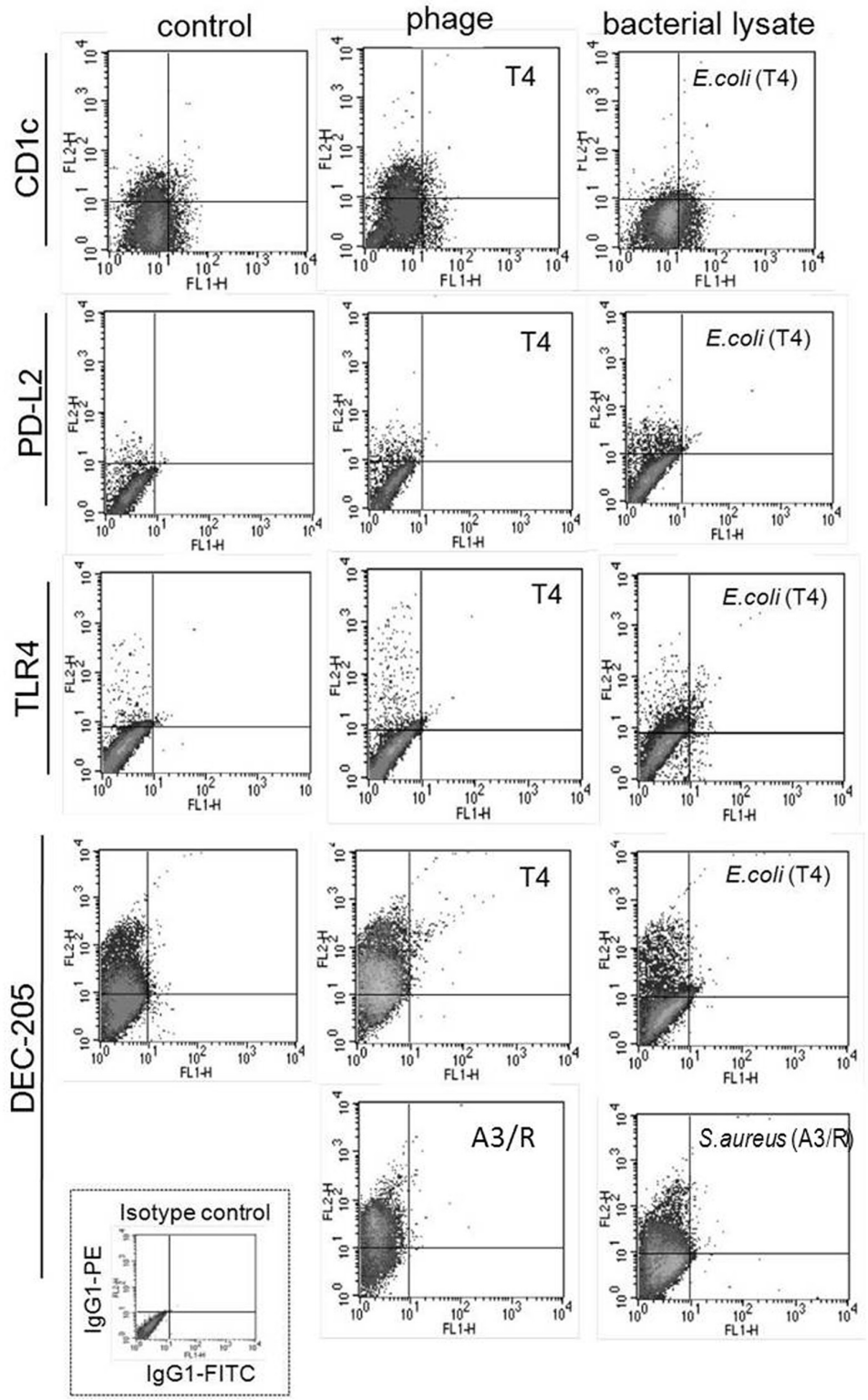

FIGURE 4 | Representative results of analysis of the expression of surface markers of mDCs differentiated in the presence of bacteriophage-generated bacterial lysates. Shown are representative density plots for markers for which statistically significant differences were found between mDCs differentiated in the presence of bacterial lysates and mDCs from control cultures (treated with PBS). In addition, the figure shows representative density plots for mDCs differentiated in the presence of bacteriophages. Anti-CD1c, anti-PD-L2, anti-TLR4, and anti-DEC-205 monoclonal antibodies were conjugated with phycoerythrine (PE). Shown is also a representative density plot for isotype control (IgG-PE).

acylated lipopeptides. CD1c was found to be capable of presenting as antigens both endogenous lipids and some components of *Mycobacterium tuberculosis* (Adams, 2014). It should be noted that $CD1c^+$ cells generated and investigated in our study are more related to monocyte-derived cells than to *bona fide* DCs (Guilliams et al., 2014).

Differences in the percentages of mDCs expressing CD1c and DEC-205, as well as in morphology of differentiating DCs suggest the presence in the lysate of T4 phage of factor(s) inhibiting the differentiation of DCs from monocytes. These factor(s) might inhibit DEC-205-mediated phagocytosis by DCs as well as presentation of lipid antigens by CD1c. However, whether the products of phage-induced lysis of bacterial cells can affect functions of DCs mediated by these molecules should be verified experimentally.

The presence of bacterial lysate of A3/R phage resulted in a decrease in the percentage of DCs expressing DEC-205 (and thus possessing a capacity for phagocytosis) and a lower number of cells with typical DC morphology.

Given that the described changes in the expression of DC markers were found only in cells cultured with phage-generated bacterial lysates, and did not occur in cells treated with purified bacteriophages, it is apparent that they were caused not by phage virions themselves, but rather by some components of bacterial cells present in the lysates. These results indicate that some products of phage-induced lysis of bacteria also could affect function of DCs *in vivo*. However, at this stage of our research we wanted to evaluate whether bacteriophage-generated bacterial lysates can have any effect on the differentiation of DCs. Further studies should be conducted to identify the component(s) of lysates that affect the expression of mDCs markers. Given that some significant changes in the expression of mDCs markers (as well as in morphology of mDCs) were induced also by *S. aureus* lysate, it is unlikely that LPS was the sole bacterial component responsible for these effects. Apart from LPS (present in *E. coli* lysate) also other bacterial components could influence the differentiation of DCs.

The interactions between the products of the phage-induced lysis of bacteria and immune cells might be important especially in the gut where phages are present in very large numbers and constitute the primary factor to regulate the growth of the microbiota (Letarov and Kulikov, 2009; Mills et al., 2013; Dalmasso et al., 2014). Lysis of bacterial cells can also occur in patients with bacterial infections treated with phage preparations. In fact, phage therapy is currently considered one of the most promising ways of treatment of antibiotic-resistant infections (Kutter et al., 2014; Wittebole et al., 2014).

The present study focused on the first stage of the development of DCs, that is the differentiation of immature DCs from monocytes. To extend knowledge on the interactions between phages and DCs, studies should be performed to evaluate the effects of phage preparations on further stages of DCs development, in particular their maturation and function of mature DCs, especially their role in the regulation of adaptive immune responses. Our results indicate that such studies should involve both purified phages and phage-generated bacterial lysates because these two types of preparations can have different effects on DCs.

AUTHOR CONTRIBUTIONS

KB performed the experiments, analyzed data, and contributed to drafting of the manuscript. JB analyzed data and contributed to drafting of the manuscript. MZ, MP and MM performed the experiments. BW-D analyzed data. GK-K conceived the study, analyzed data and contributed to drafting of the manuscript. AG conceived the study and contributed to drafting of the manuscript. All authors approved the final version of the manuscript.

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T4 Phage Tail Adhesin Gp12 Counteracts LPS-Induced Inflammation *In Vivo*

Paulina Miernikiewicz[1], Anna Kłopot[1], Ryszard Soluch[1], Piotr Szkuta[1], Weronika Kęska[1], Katarzyna Hodyra-Stefaniak[1], Agnieszka Konopka[1], Marcin Nowak[2], Dorota Lecion[1], Zuzanna Kaźmierczak[1], Joanna Majewska[1], Marek Harhala[1], Andrzej Górski[1] and Krystyna Dąbrowska[1]*

[1] *Bacteriophage Laboratory, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland,* [2] *Division of Pathomorphology and Veterinary Forensics, Department of Pathology, Wroclaw University of Environmental and Life Sciences, Wrocław, Poland*

Bacteriophages that infect Gram-negative bacteria often bind to the bacterial surface by interaction of specific proteins with lipopolysaccharide (LPS). Short tail fiber proteins (tail adhesin, gp12) mediate adsorption of T4-like bacteriophages to *Escherichia coli*, binding surface proteins or LPS. Produced as a recombinant protein, gp12 retains its ability to bind LPS. Since LPS is able to exert a major impact on the immune response in animals and in humans, we have tested LPS-binding phage protein gp12 as a potential modulator of the LPS-induced immune response. We have produced tail adhesin gp12 in a bacterial expression system and confirmed its ability to form trimers and to bind LPS *in vitro* by dynamic light scattering. This product had no negative effect on mammalian cell proliferation *in vitro*. Further, no harmful effects of this protein were observed in mice. Thus, gp12 was used in combination with LPS in a murine model, and it decreased the inflammatory response to LPS *in vivo*, as assessed by serum levels of cytokines IL-1 alpha and IL-6 and by histopathological analysis of spleen, liver, kidney and lungs. Thus, in future studies gp12 may be considered as a potential tool for modulating and specifically for counteracting LPS-related physiological effects *in vivo*.

Keywords: T4 phage, gp12, short tail fibers, lipopolysaccharide, LPS, inflammation

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****Correspondence:***
Paulina Miernikiewicz
pola@iitd.pan.wroc.pl

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INTRODUCTION

Bacteriophages that infect Gram-negative bacteria often bind to lipopolysaccharide (LPS) molecules exposed on the surface of the outer membrane. For this action bacteriophages use dedicated attachment proteins. T4-like bacteriophages adsorb to *Escherichia coli* cells by two types of specialized adhesion proteins: long tail fibers and short tail fibers. These two types of adhesins differ in their role during phage infection of bacteria, and thus they differ in the affinity to LPS. Long tail fibers consist of a few proteins that build an elongated flexible structure in which gp37 determines the affinity. Since the role of long tail fiber is "the first screening" and discrimination between susceptible and non-susceptible bacteria, its binding to bacterial receptors is reversible. Short tail fibers are known to bind LPS very firmly since their role is to anchor the phage to a host selected by long tail fibers; they assure a fixed structure during penetration of the bacterial envelope by the tail tube (Miroshnikov et al., 1998; van Raaij et al., 2001a,b; Thomassen et al., 2003).

Short tail fibers of bacteriophage T4 consist of one type of protein, gp12, that forms parallel homo-trimers. These homo-trimers attach to the phage baseplate by the N-terminus while the C-terminus functions as the LPS-binding site (van Raaij et al., 2001a,b; Thomassen et al., 2003). In the phage particle, spatial exposure of gp12 depends on the stage of infection: its LPS-binding region is initially hidden in the baseplate. To expose the LPS-binding region, another structural protein of the phage, gp10, functions as a molecular lever that rotates and extends the hinged short tail fibers, thus facilitating bacterial cell attachment. All six short fibers turn to a perpendicular position that allows for firm binding to LPS molecules (Leiman et al., 2006). Interestingly, isolated gp12, that is expressed as a recombinant protein, retains its ability to bind LPS (van Raaij et al., 2001a,b; Thomassen et al., 2003). This feature has already allowed for practical application of gp12 as an LPS ligand *in vitro*: it serves as a reactant in LPS-removal and LPS-detection kits[1].

Lipopolysaccharides, also called endotoxins, are able to exert a major impact on the immune response in animals and in humans. LPS is the prototypical pathogen-associated molecular pattern (PAMP); when detected in a living system, LPS triggers a non-specific activation. LPS is recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in both plants and animals (Poltorak et al., 1998; Alexander and Rietschel, 2001; Medzhitov, 2007). TLR4 is present on the surface of monocytes/macrophages, neutrophils, myeloid dendritic cells, mast cells, B lymphocytes, and intestinal epithelium (Sallusto and Lanzavecchia, 2002; Sabroe et al., 2005; Gerondakis et al., 2007). Recognition and immunostimulation by LPS is initialized by the combined extracellular actions of LPS binding protein (LBP), TLR4, MD-2 and CD14 the membrane-bound or soluble forms. The key step is formation of the TLR4-MD-2-LPS complex. This results in the rapid activation of intracellular signaling pathways that are highly similar to the signaling systems of IL-1 and IL-18 (Alexander and Rietschel, 2001). At the systemic level, this reaction means an acute inflammatory response, and it can be a key factor in sepsis and septic shock.

At the same time, effects of bacteriophages on functions of the immune system are gaining increasing interest in the scientific community (Górski et al., 2006, 2012; Łusiak-Szelachowska et al., 2014; Park et al., 2014; Hodyra-Stefaniak et al., 2015). This interest results from safety issues related to practical applications of bacteriophages, but also from the fact that bacteriophages have the intrinsic ability to interact with bacterial products such as LPSs and surface proteins. This ability is mediated by specific phage proteins. Therefore, here we assess LPS-binding phage protein gp12 as a potential modulator of the LPS-induced immune response. This assessment involved initial toxicity testing in cell cultures, and gp12 was further tested for its ability to interfere with LPS-induced immunostimulation *in vivo*.

[1]http://www.hyglos.de/en/products-services/products/endotoxin-removal.html (accessed December 6, 2015).

MATERIALS AND METHODS

Construction of Expression Vector

Genes encoding short tail fiber (*12*) and its chaperone gp57 (*57*) were cloned to the expression plasmid pCDF-Duet1 (Streptomycin resistance; Novagen, USA), which contains two multiple cloning sites (MCS) under a T7lac promoter control. Amplification of gene *12* was conducted using polymerase chain reaction (PCR) and T4 total DNA (Miernikiewicz et al., 2012) as a template. The primers were 12 forward 5′-AAAAGGATCCGATGAGTAATAATACATATCAACACG-3′ and 12 reverse 5′-AAAGCGGCCGCTCATTCTTTTACCTTAATTATG-3′. The *12* gene was inserted into the first multiple cloning site (MCS1) of pCDF-Duet1 via *Bam*HI and *Not*I restriction enzymes (restriction sites are underlined in primer sequences), yielding pCDFg12. Then, the *57* gene was cloned into MCS2 of pCDF-Duet1 by the GeneArt Gene Synthesis system (GeneArt, Thermo Fisher Scientific, Poland), yielding pCDFg12g57. All sequences were verified by automated Sanger sequencing (Genomed, Poland). The obtained vector pCDFg12g57 expresses gp12 with an N-terminal His-Tag and gp57 without any tags.

Expression and Purification of gp12

The pCDFg12g57 vector was expressed in a bacterial expression system. *E. coli* strain B834(DE3) F- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm met* (DE3; EMD, Europe) carrying pCDFg12g57 was grown in Luria-Bertani Broth (LB) high salt (10 g/l of NaCl) culture medium (Sigma-Aldrich, Europe or AppliChem, Europe) supplemented with streptomycin at 37°C until OD_{600} reached 0.8. Then, the bacterial culture was cooled down to 18°C, induced by 0.1 mM isopropyl thio-β-D-galactoside (IPTG) and further incubated overnight at 18°C.

Harvested bacteria were suspended in phosphate buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 8), treated with PMSF (1 mM) and incubated on ice for 15 min. The lysis was done by incubation with lysozyme (0.5 mg/ml) for 6–7 h on ice and by the freeze-thaw method (−80°C). The preparation was then supplemented with Mg^{2+} (up to 0.25 mM), DNase (10 μg/ml) and RNase (20 μg/ml), and incubated on ice for 3 h. Fractions were separated by several centrifugations (12 000 rpm, 45 min, 15°C). Soluble fractions were removed while insoluble fractions were suspended in Tris buffer (40 mM Tris–HCl pH 8, 10 mM EDTA) after each centrifugation, and gently mixed for 30 min. The procedure was repeated three times. The last pellets were suspended in phosphate buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 8) and centrifuged in the same conditions as mentioned above. Obtained soluble fractions were filtered through 0.45 μm PVDF filters and further incubated overnight at 10°C with 1 % w/v glycerol. Next, the preparation was supplemented with 25 mM imidazole and incubated with NiNTA agarose (Qiagen, Germany) at room temperature for 2–3 h. The slurry was washed with 5 L of wash buffer I (50 mM $NaH_2PO_4 \times H_2O$, 300 mM NaCl, 25 mM imidazole, 1% glycerol (w/v), 0.05% TWEEN (v/v), pH 8) and then with 5 L of wash buffer II (50 mM NaH_2PO_4 x H_2O, 300 mM NaCl, 25 mM imidazole, 1% glycerol (w/v)). These two steps

of washing were conducted with very slow flow, about 200 ml per hour. The last washing step was done with 100 ml of wash buffer III (50 mM NaH_2PO_4 x H_2O, 300 mM NaCl, 100 mM imidazole, 1% glycerol (w/v)). Protein preparations were released from the column using 5–6 sets of 5 ml of elution buffer (50 mM NaH_2PO_4 x H_2O, 300 mM NaCl, 500 mM imidazole, 4% glycerol (w/v)). The whole process was monitored by SDS-PAGE. Elutions were combined according to their purity and concentrated 2–5 times on Vivaspin centrifuge concentrators (Sartorius, Poland) at 18–20°C. Next, LPS was removed using EndoTrap Blue (Miernikiewicz et al., 2012). The final preparation was dialyzed against PBS at room temperature and filtered through 0.22 μm PVDF filters. Its concentration was determined by Lowry assay. Endotoxin level of the purified gp12 was assessed using the EndoLisa Endotoxin Detection kit (Dąbrowska et al., 2014).

Particle Size Distribution

Lipopolysaccharide was isolated from *E. coli* liquid cultures as described (Miernikiewicz et al., 2013). It was diluted with deionized water and dispersed in an ultrasonic bath. Dispersion (homogeneity) was controlled by particle size distribution measurement; particle size distribution was analyzed by the dynamic light scattering (DLS) method using Malvern Zetasizer NanoZS (Malvern Instruments, UK). The same system was used for detection of LPS-gp12 interactions: particle size distribution was measured in solutions gp12 (10 μg/ml), LPS (10 μg/ml) or a mixture of gp12 (10 μg/ml) and LPS (10 μg/ml). All samples were diluted with deionized water and offset for 1 min prior to each measurement. Albumin was used as a control. All experiments were done at 25°C. The measurements were repeated five times.

Cytotoxicity

The influence of gp12 on growth and viability of murine and human cells was investigated. Tests were done on murine fibroblasts (Balb3T3) and on human skin microvascular endothelial cells (HSkMEC). Cells (10^4 cells/well) were grown in 96-well plates for 24 h at 37°C, 5% CO_2. Then, cells were supplemented with various concentrations of gp12 (1, 10, 100 μg/ml) and incubated for 72 h. PBS treated cells served as a normal cell growth control (solvent control). Next, the sulforhodamine B (SRB) test was done. Briefly, 50 μl of 50% trichloroacetic acid was added to each well and incubated for 1 h at 4°C. Then, plates were washed five times with H_2O, treated with 50 μl of 0.4% sulforhodamine and incubated for 30 min at room temperature. 1% acetic acid was used to wash plates and 10 nM Tris to dissolve precipitated sulforhodamine. Measurement of cell viability was done at 540 nm using a microplate reader (Gen5, Data Analysis Software).

Animal Model

All animal experiments were performed according to EU Directive 2010/63/EU for animal experimentations and were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wrocław, Poland. The female C57Bl6/J (6–10 weeks) mice were purchased from the Center of Experimental Medicine, Medical University of Białystok, Poland, and bred under specific pathogen free (SPF) conditions in the Animal Breeding Center of the Institute of Immunology and Experimental Therapy (IIET).

Female C57Bl6/J mice ($N = 6$) were injected intraperitoneally with 100 μg/mouse of gp12, 1 mg/kg of LPS or 100 μg/mouse of gp12 simultaneously with 1 mg/kg of LPS. Control mice were inoculated with PBS. After 3 and 7 h murine blood was collected from the tail vein, whilst after 24 h murine blood was collected from the orbital plexus vein into heparinized tubes. All bleeding procedures were done

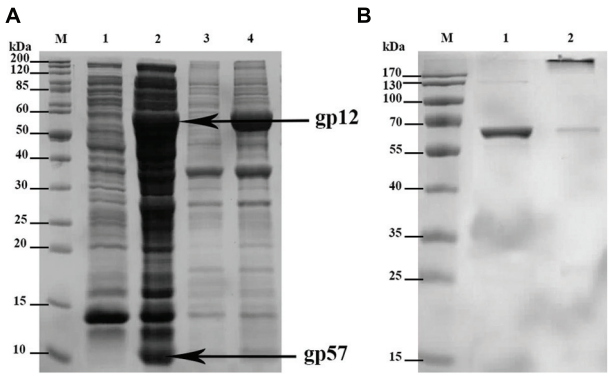

FIGURE 1 | SDS-PAGE of: (A) expressed recombinant His-tagged gp12 and gp57; overexpressed proteins are marked with arrows; M-molecular weight marker; 1-soluble fraction of the culture before induction (control); 2-soluble fraction of the culture after induction (expression); 3-insoluble fraction of the culture before induction (control); 4-insoluble fraction of the culture after induction (expression). **(B)** gp12 final preparation as trimeric and monomeric; M – molecular weight marker; 1 – heated gp12 (denatured protein) as monomeric (57.5 kDa); 2 – non-heated gp12 as trimeric (172.5 kDa).

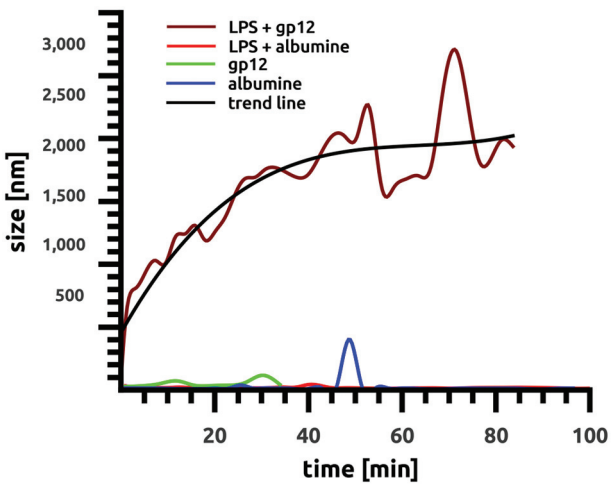

FIGURE 2 | Binding of gp12 to lipopolysaccharide *in vitro* over time. Binding was identified as changes in average particle size in solution by the dynamic light scattering (DLS) method using Malvern Zetasizer NanoZS (Malvern Instruments, UK). All samples were diluted with deionized water, and size distribution was measured at 25°C.

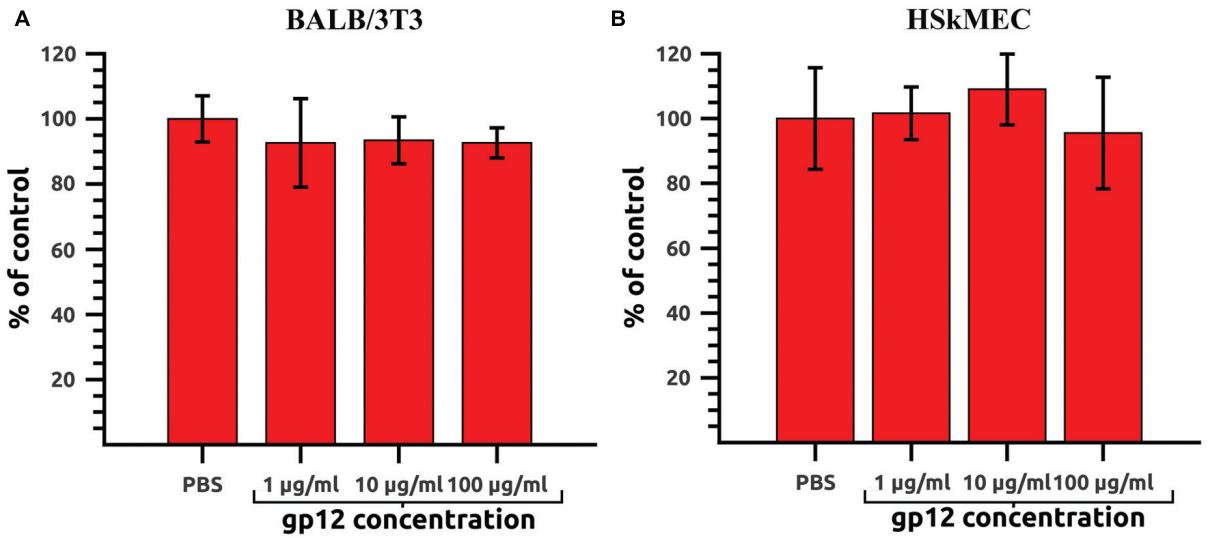

FIGURE 3 | Proliferation of gp12-treated mammalian cells *in vitro*. (A) Murine fibroblasts (BALB/3T3) treated with 1, 10, or 100 µg/ml of gp12 for 72 h. PBS served as a control. **(B)** Human skin microvascular endothelial cells (HSkMEC) treated with 1, 10, or 100 µg/ml of gp12 for 72 h. PBS served as a control.

under anesthesia. Serum was separated from the blood by double centrifugation at 2250 × *g* and used for the ELISA assay. Then animals were sacrificed by cervical dislocation and the following organs were excised, for histopathological examination: lungs, liver, spleen, and kidneys. All experiments were repeated three times. A representative experiment is presented.

Cytokine Assay

The progress of the inflammatory reaction in the murine blood was monitored. Concentrations of IL-1α and IL-6 were measured by commercially available ELISA kits (PeproTech). Mean values per group of animals are presented.

Histopathology

For histopathological examination, the murine organs (lungs, liver, spleen, and kidneys) were fixed in 7% formaldehyde for 48 h. Later, samples were embedded in paraffin blocks. Histological slides of 4 µm thickness were prepared and counterstained with hematoxylin and eosin (HE), and examined microscopically. All sections were analyzed by researchers blind to the experimental groups of the samples.

Statistics

Statistical analysis was done by ANOVA, the Kruskal–Wallis test and ANOVA with Bonferroni and Holm multiple comparison calculation, with significance level $p = 0.05$. The Statistica 8.0 software package was applied (StatSoft, Inc. STATISTICA data analysis software system, version 8.0)[2].

[2]StatSoft, Inc: STATISTICA (data analysis software system), version 8.0. Available at: http://www.statsoft.com (accessed April 20, 2015).

RESULTS

Expression and Purification of gp12

The short tail fibers of bacteriophage T4 are well known for their LPS binding capability. This ability depends, however, on correct gp12 quaternary structure meaning that phage adhesin has to form its trimeric structure to be able to bind LPS. Proper folding of gp12 was reported only in the presence of another T4 phage protein, gp57. Its function as a molecular chaperone cannot be substituted by host protein overexpression, e.g., GroEL/ES. Gp57 increases folding efficacy and production efficiency of gp12, and it concurrently inhibits insoluble aggregate formation by this phage adhesin (Burda and Miller, 1999). Thus, we cloned genes *12* and *57* of T4 phage to pCDF-Duet1, the expression vector that enables simultaneous production of two proteins in bacterial expression systems (**Figure 1A**).

Co-expression of gp12 with its natural chaperonin gp57 resulted in proper trimeric structure of the obtained product as verified by SDS-PAGE. Verification was done according to King and Laemmli (1971), who demonstrated that properly folded, trimeric gp12 resisted dissociation by SDS at ambient temperature, whereas heating caused monomerization of the polypeptide chains (King and Laemmli, 1971). The molecular weight of trimeric gp12 is 172.5 kDa, while that of monomeric gp12 is 57.5 (**Figure 1B**).

Binding of gp12 to Lipopolysaccharide *In Vitro*

The ability of purified recombinant gp12 to bind LPS was tested *in vitro* by particle size distribution measurement in LPS solution. This method employed DLS, and it allowed for detection of a relative increase in average size of aggregating molecules in solutions. Purified gp12 solution as well as LPS solution was

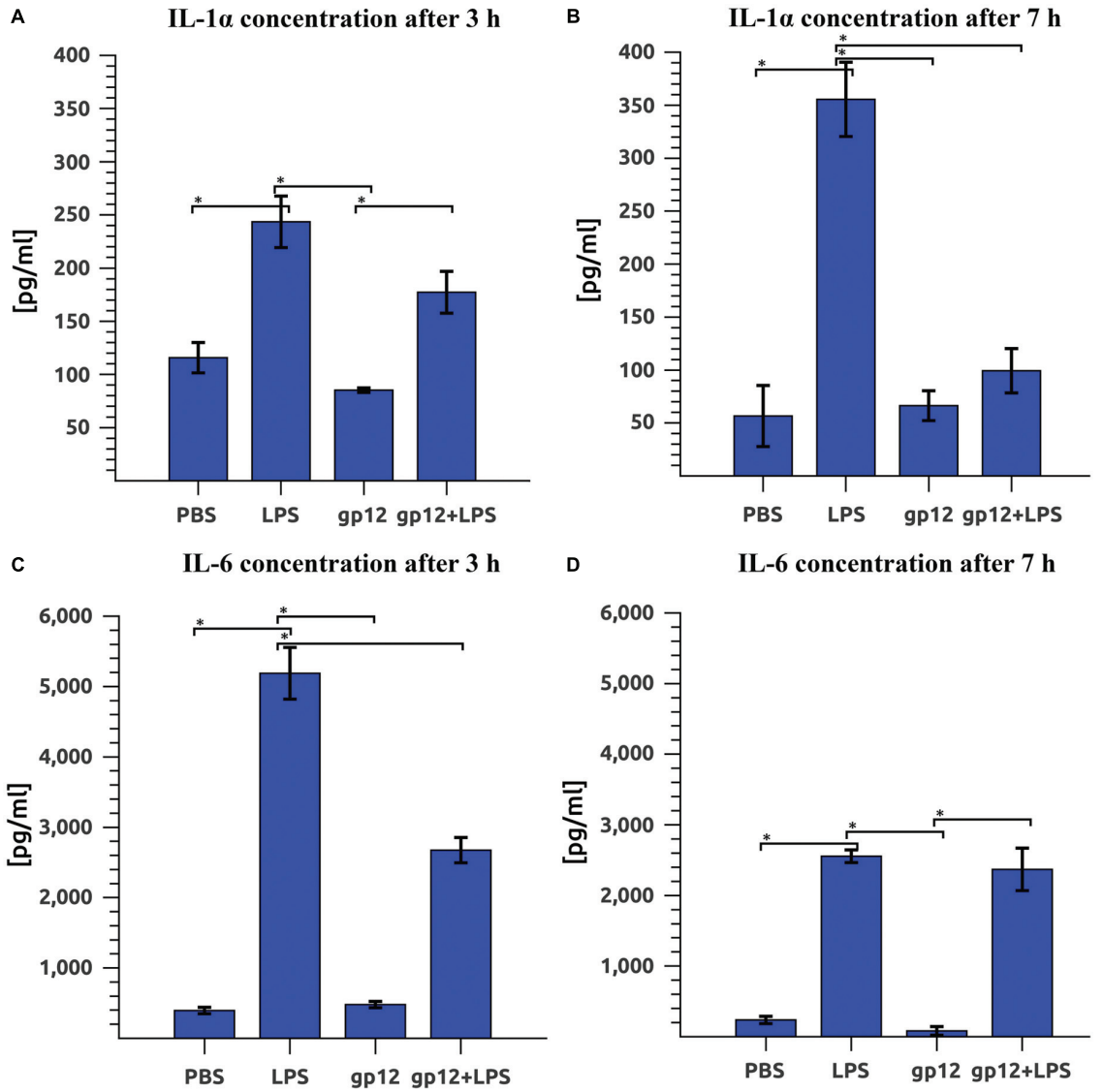

FIGURE 4 | Effect of phage protein gp12 on: (A) IL-1α concentration in mice 3 h after treatment. (B) IL-1α concentration in mice 7 h after treatment; **(C)** IL-6 concentration in mice 3 h after treatment; **(D)** IL-6 concentration in mice 7 h after treatment. *$p = 0.05$.

dominated by small molecules, 51 nm and 95.45 nm, respectively; these values were stable in time. However, after mixing gp12 with LPS the average size of molecules in the solution markedly increased to 1980 nm (**Figure 2**). These results suggest that recombinant gp12 was able to bind and form complexes with LPS. Formation of these complexes was clearly visible within a few minutes, and it seemed to be stabilized after approximately 45 min, with the average diameter of complexes being approx. 2000 nm (**Figure 2**).

Safety Testing of gp12 on Cell *In Vitro* Cultures

Recombinant gp12, intended for *in vivo* application, was first tested on mammalian cell lines *in vitro* to exclude its direct toxic effects on cells. Potential harmful effects were assessed in a proliferation assay on murine fibroblasts (BALB/3T3) and HSkMEC. Both cell lines were treated with gp12 in cultures (doses: 100, 10, or 1 μg of the tested protein per ml). After 72 h of incubation, cell viability was assessed by the SRB method. No toxic or antiproliferative effects of gp12 were detected in the cells (**Figures 3A,B**).

Effect of gp12 on LPS-Induced Inflammation *In Vivo*

The effect of gp12 on LPS-induced inflammation *in vivo* was investigated in mice by comparison of serum inflammatory markers (cytokines). Cytokines that are typical markers of inflammation induced by LPS are IL-1α and IL-6. Here we

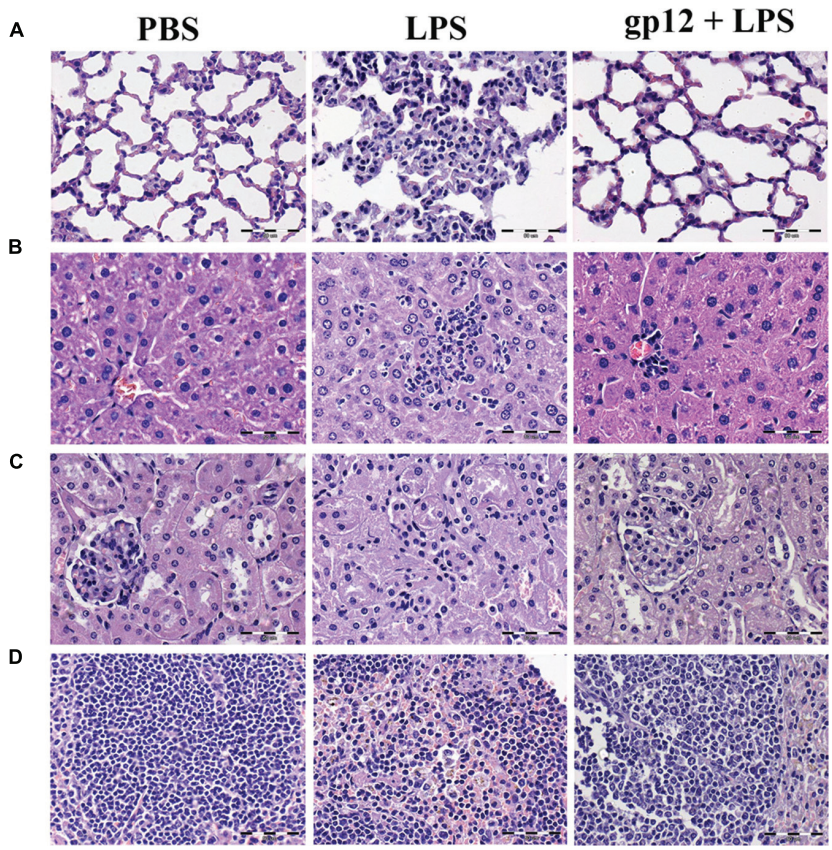

FIGURE 5 | Histopathological examination of selected animal tissues. (A) lungs; **(B)** liver; **(C)** kidneys; **(D)** spleen.

compared IL-1α and IL-6 in mice treated with LPS (1 mg/kg) or with purified gp12 (100 μg/mouse) or treated both with LPS (1 mg/kg) and purified gp12 (100 μg/mouse). Control mice were treated with PBS.

Immediate application of gp12 to mice challenged with LPS resulted in a small reduction of serum IL-1α 3 h after the treatment (27% reduction, insignificant) and in a substantial reduction 7 h after the treatment: 72% ($p = 0.002$). In this case, the serum level of IL-1α in mice challenged with LPS and treated with gp12 was similar to that observed in control mice (**Figures 4A,B**). The second marker of inflammation, IL-6, was also reduced in LPS-challenged mice treated with gp12 (in comparison to non-treated animals). This reduction was 48% ($p = 0.001$) 3 h after the treatment; however, no important effect was observed 7 h after the treatment (**Figures 4C,D**). 24 h after the challenge with LPS no significant differences were noted, while serum levels of the tested cytokines were normalized after that time (data not shown). Importantly, in mice treated with gp12 alone no pro-inflammatory activity of this protein was observed and no visible adverse effects of the treatment were noted in these animals (**Figure 4**).

Further, leukocyte infiltration into selected tissues was examined in these animals by histological microscopy (24 h after the induction). As presented in **Figure 5**, infiltration of leukocytes to lungs, liver and spleen was markedly increased in mice treated with LPS when compared to control (PBS treated) mice, which indicated an inflammatory reaction of the immune system. However, in mice treated with recombinant gp12 immediately after LPS injection, leukocyte infiltration in the investigated tissues was minor (**Figure 5**). Gp12 significantly decreased inflammatory infiltrate induced by LPS in liver and spleen (**Figure 5**). No harmful effects of gp12 was observed in tissues of mice treated with gp12 alone. These observations suggest that gp12 may counteract the pro-inflammatory effect of LPS *in vivo*.

DISCUSSION

Bacteriophage T4 tail adhesin gp12 was produced in native conformation as a recombinant protein in a bacterial expression system with the chaperone gp57. Similarly to its natural prototype, this protein was able to form trimers and to bind LPS *in vitro*, as shown by DLS (**Figure 2**). Such a product was not harmful for cell proliferation in cell cultures *in vitro*, nor were there any evident harmful effects on living mice (100 μg/mouse). It was further used in combination with LPS in a murine model to assess whether binding of gp12 to LPS might decrease the ability of LPS to induce inflammation *in vivo*. As revealed by serum cytokine assay and by examination of leukocyte infiltration into

spleen, liver, kidney, and lungs, gp12 was able to counteract the inflammatory response to LPS *in vivo*.

LPS consists of a poly- or oligosaccharide region that is anchored in the outer bacterial membrane by a specific carbohydrate lipid moiety termed lipid A. The lipid A component is commonly considered as the primary immunostimulatory center of LPS (Alexander and Rietschel, 2001) while phage short tail fibers bind to the core region of LPS (van Raaij et al., 2001a,b). However, immunoactivation by LPS in mammalian systems depend on the LPS form. One can find "classical" strongly agonistic (highly endotoxic) forms of LPS, but several have been identified that display comparatively low or even no immunostimulation for a given mammalian species. Some members of the latter more heterogeneous group are capable of antagonizing the effects of strongly stimulatory LPS/lipid A forms (Alexander and Rietschel, 2001). These observations suggest that details of LPS structure determine its ability to induce an inflammatory response. In light of this, binding of gp12 to the LPS molecule may affect the LPS structure sufficiently to decrease its ability to induce immunostimulation.

Moreover, the TLR4-MD-2-LPS complex has been crystallized and its structure has been determined at 3.1 Å resolution by Park et al. (2009), thus revealing involvement of the whole LPS molecule in the reaction with the mammalian receptor complex. Binding of LPS induces the formation of a symmetrical dimer of two TLR4-MD-2-LPS complexes. The LPS bound to MD-2 directly bridges the two TLR4 molecules. Five of the six lipid chains in LPS are completely buried inside the MD-2 pocket, but the sixth is partially exposed to the surface of MD-2 and forms a hydrophobic interaction with TLR4. Hydrophilic side chains in the surrounding regions of MD-2 and TLR4 support the hydrophobic core of the interface by forming hydrogen bonds and ionic interactions (Gay and Gangloff, 2007; Kim et al., 2007; Park et al., 2009). The molecular weight of gp12 (56 kDa) is considerable in comparison to TLR4 (approx. 95 kDa). We hypothesize that binding of phage protein gp12 to the hydrophilic core disturbs its function in formation of TLR4-MD-2-LPS complexes, and may preclude a signaling pathway that could lead to immunostimulation.

We propose interaction of the phage tail adhesin gp12 with LPS as a potential modulator of LPS-induced inflammatory effects. It is necessary to emphasize that sepsis or septic shock may have different etiology. However, in many cases LPS is the key factor triggering harmful physiological processes. Elucidation of structure-activity correlations in LPS has contributed to understanding of both immunostimulatory and toxic septic processes, and it allowed for development of new pharmacological and immunostimulatory strategies in infectious and malignant diseases. Thus, in future studies gp12 may be considered as a potential tool for modulation and specifically for counteracting LPS-related physiological effects *in vivo*.

AUTHOR CONTRIBUTIONS

PM planned the experiments, executed data processing, and analysis, participated in writing the manuscript, executed most of the laboratory work: gene cloning, protein expression and purification, LPS preparation, dynamic light scattering, protein toxicity testing *in vitro*, development and testing in animal model, cytokine tests. WK and AnK participated in protein expression and purification, cytokine testing and work with animals. RS participated in protein expression and purification, LPS preparation and dynamic light scattering. PS participated in protein expression and purification and in protein toxicity testing *in vitro*. MN executed and analyzed histopathological assay. KH-S, AgK, DL, ZK, and JM participated in laboratory work, in work with animals and in data processing. MH prepared graphics presented in the manuscript. AG consulted immunological aspects of the work and reviewed the manuscript. KD guided and supervised the work and analysis of results, wrote the manuscript, participated in laboratory work and in work with animals.

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Antibody Production in Response to Staphylococcal MS-1 Phage Cocktail in Patients Undergoing Phage Therapy

Maciej Żaczek[1], Marzanna Łusiak-Szelachowska[1], Ewa Jończyk-Matysiak[1], Beata Weber-Dąbrowska[1,2], Ryszard Międzybrodzki[1,2,3], Barbara Owczarek[1], Agnieszka Kopciuch[1], Wojciech Fortuna[1,2], Paweł Rogóż[2] and Andrzej Górski[1,2,3]*

[1] Bacteriophage Laboratory, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland, [2] Phage Therapy Unit, Medical Center of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland, [3] Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland

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***Correspondence:**
Maciej Żaczek
zaczek@iitd.pan.wroc.pl

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In this study, we investigated the humoral immune response (through the release of IgG, IgA, and IgM antiphage antibodies) to a staphylococcal phage cocktail in patients undergoing experimental phage therapy at the Phage Therapy Unit, Medical Center of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland. We also evaluated whether occurring antiphage antibodies had neutralizing properties toward applied phages (K rate). Among 20 examined patients receiving the MS-1 phage cocktail orally and/or locally, the majority did not show a noticeably higher level of antiphage antibodies in their sera during phage administration. Even in those individual cases with an increased immune response, mostly by induction of IgG and IgM, the presence of antiphage antibodies did not translate into unsatisfactory clinical results of phage therapy. On the other hand, a negative outcome of the treatment occurred in some patients who showed relatively weak production of antiphage antibodies before and during treatment. This may imply that possible induction of antiphage antibodies is not an obstacle to the implementation of phage therapy and support our assumption that the outcome of the phage treatment does not primarily depend on the appearance of antiphage antibodies in sera of patients during therapy. These conclusions are in line with our previous findings. The confirmation of this thesis is of great interest as regards the efficacy of phage therapy in humans.

Keywords: antibodies, antimicrobials, ELISA, immune response, phage cocktail, phage therapy, Staphylococcal Infections

INTRODUCTION

Phage treatment is considered one of the most promising therapies in fighting human pathogenic bacterial strains, including those that are antibiotic resistant such as methicillin-resistant *Staphylococcus aureus* (MRSA). Due to constantly declining effectiveness of antibiotics, pathogenic bacteria have become endemic organisms, especially in medical care units (Arnold et al., 2011; Cheon et al., 2016). Regardless of such worldwide increasing microbiological hazard, the wider

use of phages faces skepticism over its effectiveness, as it was postulated that human antibodies may have a negative effect on favorable treatment results (Sulakvelidze et al., 2001). Herein, we estimate the induction of antiphage antibodies and their potential neutralizing influence on *S. aureus* MS-1 phage cocktail.

Data regarding the antiphage humoral response during phage treatment are still extremely scarce. Only a few articles describe antibacteriophage activity of human sera of patients during phage treatment and healthy volunteers (Kucharewicz-Krukowska and Ślopek, 1987; Bruttin and Brussow, 2005; Górski et al., 2007; Łusiak-Szelachowska et al., 2014). Kucharewicz-Krukowska and Ślopek (1987) reported that induction of antiphage antibodies was detected in 54.4% of patients during therapy (the 10th day of phage treatment, oral administration). Only in 3 of 57 patients (5.3%) did sera indicate high antiphage activity. Similarly, in the experiment by Bruttin and Brussow (2005), none of the examined volunteers showed an increased level of antiphage antibodies (IgG, IgA, and IgM) after oral administration. Among 122 patients from the Phage Therapy Unit in Wrocław, only 15 of them (12.3%) demonstrated high ($K > 18$) antiphage activity of sera (AAS), mostly during local administration (Łusiak-Szelachowska et al., 2014). The same report showed no clear correlation between phage administration and increased level of antiphage antibodies in patients' sera evaluated by absorbance measurements using the ELISA test. Further studies (Łusiak-Szelachowska et al., 2016) clearly showed that AAS depends on the route of phage administration. All patients receiving staphylococcal phage preparations orally showed a low level of AAS, whilst those with local administration of phage cocktail had high AAS in almost half of the cases. Generally, use of a phage cocktail resulted in a stronger immune response than monotherapy. With regard to the relatively small number of patients with such high activity of sera, it is difficult to define the relationship between serum antiphage activity, the level of antiphage antibodies and effectiveness of the phage therapy. It has to be said that induction of antiphage antibodies and their binding to phage antigens does not necessarily mean the loss of phage viability (Górski et al., 2012).

Our group (Łusiak-Szelachowska et al., 2014) published probably the first report where the production of antiphage antibodies was compared to their neutralizing properties and was related to the clinical outcome in patients receiving phage therapy. The results shown below are a continuation of the previous research. Both papers allow one to determine whether phage therapy induces production of neutralizing antiphage antibodies and whether they are associated with the results of the treatment.

MATERIALS AND METHODS

Ethics Approval Statement

Experimental phage therapy was approved by the Bioethics Committee at the Wrocław Medical University (approval number KB-349/2005 with further amendments) and was conducted in accordance with the Declaration of Helsinki and national rules governing experimental therapy. Each patient gave informed consent prior to beginning the treatment. The study was approved by the same bioethical commission (approval number KB-414/2014).

Patients Subjected to Phage Therapy and Healthy Volunteers

Adult patients with various infections (e.g., bone infections, sinus infections) resistant to antibiotic treatment received phage treatment under the therapeutic protocol entitled "Experimental phage therapy of drug-resistant bacterial infections, including MRSA infections" (Międzybrodzki et al., 2012). Patients ($n = 20$) treated in the Phage Therapy Unit in Wrocław, Poland with the *S. aureus* MS-1 phage cocktail were examined. Nineteen of them suffered from infections caused by methicillin-sensitive *Staphylococcus aureus* strains (MSSA); from one patient the *Staphylococcus lugdunensis* strain was isolated. All patients received treatment over the years 2012−2015. Specific data regarding patients examined in this study are summarized in **Table 1**. Sera of 10 healthy blood donors came from the Blood Donation Center, Clinical Military Hospital in Wrocław, Poland.

The effectiveness of phage treatment was evaluated according to the scale from A to G (Międzybrodzki et al., 2012) where categories A-C were considered as good responses to the phage therapy (A – pathogen eradication, B – good clinical results, C – clinical improvement) and categories D-G were considered as inadequate responses to phage treatment (D – questionable clinical improvement, E – transient clinical improvement, F – no response to the treatment, G – clinical deterioration).

The details of patient treatment including routes of administration in different types of infections have been described earlier (Międzybrodzki et al., 2012).

Phage Preparations

Testing Bacterial Susceptibility to Phages

The MS-1 phage cocktail consists of three lytic *S. aureus* phages – 676/Z, A5/80 and P4/6409. Among 458 tested MSSA strains of *S. aureus*, 73.4% were susceptible to the MS-1 phage cocktail. Effectiveness was lower for MRSA strains (from 28 tested strains 53.6% of them were susceptible to MS-1 phage cocktail). Detailed analysis of susceptibility to the MS-1 phage cocktail is presented in **Table 2**. The phage components of MS-1 cocktail were chosen to achieve optimal (maximal) lytic range based on the theoretical statistical analysis of the results of the sensitivity of staphylococcal strains against individual staphylococcal phages from our collection. The results shown in **Table 2** are presented for new collection of staphylococcal strains isolated from patients after introduction of MS-1 phage cocktail. Therefore, they represent a real staphylococcal sensitivity to MS-1 as well as to its individual phages. We are working on new, more efficient composition of staphylococcal phage cocktail based on the results and experience obtained from using MS-1. All our patients are treated with targeted phage preparations, which means that in each case we use only phages or phage cocktail which are lytic against a pathogenic bacterial strain isolated from the patient. Therefore, in all our MRSA cases treated with MS-1, the bacteria were susceptible to the phage cocktail. Otherwise, our

TABLE 1 | List of examined patients from the Phage Therapy Unit in Wrocław, Poland.

Patient	Type of infection	Preparation used in phage therapy	Route of administration
1	Left shank infection	OP MS-1	Locally
2	Right hip joint fistula	MS-1	Locally
3	Chronic infection of the paranasal sinuses and the maxillary sinus	MS-1	Locally and orally
4	Ulceration of perianal area after colon resection	MS-1, since 77th day of therapy *S. aureus* A5/80 phage, after 5-month interruption *S. aureus* φ200 phage	All phages were applied locally and orally
5	Inflammation of the left tibia	MS-1	First locally, after 8 weeks of the treatment orally
6	Infection of the subcutaneous tissue with a thigh fistula	MS-1, since 105th day of therapy *S. aureus* 676/F phage	all phages were applied orally
7	Left elbow infection with active fistula	MS-1	Locally
8	Periprosthetic infection of the left hip	OP MS-1 TOP, since 42nd day of therapy *S. aureus* φ200 phage	OP MS-1 TOP phage cocktail was applied locally, φ200 phage orally
9	Inflammation of the left tibia	MS-1	Locally
10	Fracture of the ankle with ulceration	MS-1, after 8-month interruption *S. aureus* 676/F phage	Locally, after 8.5 months of the treatment orally, 676/F phage was applied only orally
11	Chronic infection of the paranasal sinuses and the throat	MS-1	locally and orally
12	Inflammation of the right calcaneus	MS-1	Locally
13	Right foot infection	MS-1	Locally
14	Chronic infection of the paranasal sinuses	MS-1, since 35th day of therapy *S. aureus* P4 phage	Orally, P4 phage was applied locally and orally
15	Chronic conjunctivitis, chronic sinusitis	MS-1, after two months treatment *S. aureus* A3/R phage	all phages were applied Locally and orally
16	Periprosthetic infection of the right hip	MS-1	Locally
17	Inflammation of the right femur	MS-1	Locally
18	Surgical wound infection of the belly with fistula	MS-1	Orally
19	Right thigh infection	MS-1	Locally
20	Chronic purulent inflammation of the right tibia	MS-1	Locally

TABLE 2 | Susceptibility of *S. aureus* MSSA and MRSA strains to MS-1 phage cocktail (Weber-Dąbrowska et al., 2012; unpublished data).

	MS-1	676/Z	A5/80	P4/6409
Susceptible (MSSA)	336 (73.4%)	260 (72.8%)	252 (70.6%)	192 (53.8%)
Resistant (MSSA)	122 (26.6%)	97 (27.2%)	105 (29,4%)	165 (46.2%)
TOTAL	458 (100%)	357 (100%)	357 (100%)	357 (100%)
Susceptible (MRSA)	15 (53,6%)	4 (57.1%)	3 (42.9%)	2 (28.6%)
Resistant (MRSA)	13 (46,4%)	3 (42.9%)	4 (57.1%)	5 (71.4%)
TOTAL	28 (100%)	7 (100%)	7 (100%)	7 (100%)

MRSA patients were treated with another single staphylococcal phage from our collection. If we were not able to identify an active phage, patients were not qualified to experimental phage treatment.

Bacterial susceptibility to specific polyvalent staphylococcal phages from our collection was evaluated by spotting method (Ślopek et al., 1983; Chirakadze et al., 2009). The bacteria cultures were prepared in liquid broth medium. Next, the suspensions were spread on plates with solid agar medium. Plates were dried in incubator (37°C, 1.5 h). Drop of each phage lysate (10^7-10^9 pfu/ml of initial suspension) was spotted on plate surface, then plates were incubated (37°C, 6 h) and stored at 4°C until the following day. A positive result was recognized (under the therapeutic point of view) when confluent or semi-confluent lysis was observed.

Phage Propagation

All phages used in this study were propagated in liquid broth (LB). To each of five flasks, containing 160 ml of medium, 5 ml overnight host bacterial culture was added, mixed together and incubated for 1 h at 37°C. At the same time one large flask (3—5 l capacity) was filled with a mixture of LB (160 ml), 5 ml of high titre phage stock (at least 10^8-10^9 pfu/ml) and 5 ml of overnight host strain culture. After an 1 h incubation period the content of one of the five flasks was added to a large flask. This step was repeated every 30 min until the last flask was emptied. From

the moment of the phage addition the culture was incubated for 10 h at 37°C. After this step the large flask was stored under refrigeration at 4°C. The following day, phage culture was filtered aseptically using membrane filters (0.22 μm) in a laminar flow cabinet and its titer was determined.

Therapeutic Phage Preparations

All examined patients (with two exceptions) received phage cocktail lysates (MS-1) produced by IBSS BIOMED S.A. in Kraków, Poland. According to the leaflet, the therapeutic phage dose was at least 5×10^5 pfu/ml. One patient received a purified phage cocktail (OP MS-1) with a phage dose of at least 1×10^9 pfu/ml of each phage suspended in phosphate buffered saline (PBS) with 10% addition of saccharose. Finally, one patient received an analogous purified phage cocktail deprived of saccharose (OP MS-1 TOP). Saccharose is used to maintain the stability of storage conditions, including structure preservation and specific interactions (Chang et al., 2005). Typically, phages were applied orally and/or locally 2−3 times a day in the amount of 5−10 ml per dose (each case was individually evaluated by a physician). Before each oral phage administration 10 ml of oral suspension of dihydroxyaluminium sodium carbonate (68 mg/ml) was applied as well.

Purified Phage Preparations

Due to high sensitivity of the ELISA technique, all phage preparations used in our examinations (676/Z, A5/80, and P4/6409) were purified according to a previously described method (Łusiak-Szelachowska et al., 2014) with some changes. After phage propagation in LB liquid medium, phage lysates were concentrated using Vivaflow 200 (tangential flow module) with Hydrosart membrane (the molecular weight cut-off is 30 000). The next step was removal of lysate contaminants from the phage suspension by size exclusion chromatography using a Sepharose CL-4B column (GE Life Sciences 26/100) under the following conditions: elution buffer 0.068 M phosphate buffer, pH 7.2; flow rate 2.2 ml/min; detection UV at 280 nm. Sepharose CL-4B is a cross-linked form of Sepharose, which is chemically and physically more resistant than Sepharose itself and offers better flow characteristics with the same selectivity. Purified phage particles were dialysed to PBS. The final titers ranged from 1.3×10^{10} to 3.5×10^{10} pfu/ml. To evaluate the level of endotoxins, the Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) test was performed, according to the manufacturer's instructions (Lonza). The LAL test is a quantitative test mainly for Gram-negative bacterial endotoxins. However, it is helpful to detect endotoxins in every phage preparation. As our findigs revealed, LPS can be present even in sterile peptone water. It happens because we use reusable glass tubes and LPS is well known for its very strong adhesion properties. Even relatively low dose of LPS at the beginning of phage propagation and purification is multiplied during the concentration process. The final levels of detected endotoxins in phage preparations varied from 994 to 1727 EU/ml, which gave no more than 20 EU/ml after dilution of phage preparations for ELISA purposes. Similar low values were detected in staphylococcal phage preparations used in phage therapy.

Preparation of Serum Samples

All blood samples were taken before and during treatment, in some cases also after phage therapy. The sera were separated from heparinized blood samples by centrifugation at 1,500 *g* for 10 min and stored in 1−1.5 ml aliquots in a freezer at −70°C.

Phage Inactivation

The phage neutralization by human sera was estimated as the rate of phage inactivation (*K* rate) by the method described in our previous work (Łusiak-Szelachowska et al., 2014). Fifty microliter of phage lysate (1×10^6 pfu/ml) was added to 450 μl of diluted serum (from 1:10 up to 1:1500). Next, sample was incubated at 37°C for 30 min. After incubation the mixture was diluted 100 times with cold broth and the phage titer was determined. *K* rate was estimated using the equation:

$$K = 2.3 \times (D/T) \times log(P0/Pt) \quad (1)$$

where, *K* is the rate of phage inactivation, *D* is the reciprocal of the serum dilution, *T* stands for the time in minutes during which the reaction occurred (30 min. in this case), P0 is the phage titer at the start of reaction and Pt is the phage titer after reaction. A *K* rate less than 5 was considered as weak phage neutralization, between 5 and 18 as a medium level, and above 18 as a high level of phage neutralization.

ELISA Procedure

Immune analysis was based on detection of the level of specific antiphage antibodies in human sera reacting with phage antigens using an indirect ELISA technique. We used three different types of secondary antibody to detect specifically human IgG, IgA, and IgM. In contrary to our previous results based only on raw absorbance values (Łusiak-Szelachowska et al., 2014), herein we established standard reference serum and final results have been provided as antibody units (AU). The immunological response was measured to each phage separately (*S. aureus* 676/Z, A5/80, and P4/6409 phages) as well as to the entire *S. aureus* MS-1 phage cocktail. Purified *S. aureus* phage preparations as antigens were diluted immediately before use in 0.05 M coating buffer (carbonate−bicarbonate buffer; Sigma−Aldrich) to obtain the titer at 5×10^8 pfu/ml. The phage cocktail was prepared after diluting phages in coating buffer by mixing equal volumes of three different *S. aureus* phage solutions (676/Z, A5/80, and P4/6409).

At the beginning of the procedure, 96-well flat-bottom microplates (Nunc MaxiSorp; Thermo Scientific) were loaded with 100 μl of purified phage preparations as antigen-containing samples. Next, covered microplates were incubated for 1 h at 37°C and washed six times using PBS with 0.05% addition of Tween 20 (Sigma−Aldrich), which is a synthetic detergent helpful in removing non-binding residue antigens. Multiple washing was a mandatory stage in the procedure after each incubation period at 37°C. The washing step was performed by an automatic 96-needle microplate washer (HydroSpeed; Tecan) with a wash rate at 350 μl/s. The next step was to apply to microplate wells 200 μl of blocking protein (1% solution of casein sodium salt from bovine milk in PBS; Sigma-Aldrich) for

blocking any non-specific binding sites. Microplates were then again incubated for 1 h at 37°C. As primary antibodies human serum samples, diluted in blocking solution with 0.05% addition of Tween 20 in the proportions 1:1000 and 1:10 000, were used. Each serum sample was applied in duplicate by pipetting 100 μl of the solution per well. After that, microplates were incubated for 1 h at 37°C. Specific antiphage antibodies bound with antigen were detected by secondary antibodies. We used three different secondary antibodies produced in goat linked with the enzyme horseradish peroxidase (HRP) binding human IgG, IgA or IgM (Sigma−Aldrich). All anti-human secondary antibodies were diluted in blocking solution with 0.05% addition of Tween 20 in the proportion 1:15 000 to obtain working concentrations at ∼0.5 μg/ml. Microplates were loaded with 100 μl/well of secondary antibody solution and again incubated for 1 h at 37°C. The final step was to apply 200 μl/well of a chemical substrate (*o*-Phenylenediamine; Sigma−Aldrich) suspended in 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma−Aldrich) that is converted by the enzyme into a color measured spectrophotometrically to determine the presence and quantity of antiphage antibodies. Microplates with a substrate were incubated at room temperature for 30 min in the dark. After the incubation period, microplates were read on a multiwell plate reader (Sunrise; Tecan) using Magellan 7.1 software at 450 nm with shaking immediately before the reading. One series of the whole procedure closed at 1 day. We avoided night storage of loaded microplates in refrigerated conditions as low temperatures had an influence on the repeatability of the results. The value of absorbance in blank wells (filled only with buffer) was subtracted from the tested samples.

To verify the reliability of the tests, a series of control samples was carried out on each microplate to detect possible errors. To evaluate the blocking properties of casein solution as well as to detect any non-specific binding of human antiphage antibodies (IgG, IgA, IgM) on microplate surface some wells were loaded with coating buffer (without phages as antigens). To detect unusual cross-reactivity between the antigen and the secondary antibody, some microplate wells contained a control human serum sample deficient in IgG, IgA and IgM (Sigma−Aldrich). Control serum was prepared according to the manufacturer's instructions in the same solution as all human serum samples. Some wells were loaded only with secondary antibody and substrate to detect any unusual endogenous reaction product. Of note, none of the above-mentioned control samples ever gave higher values of absorbance than blank wells. This means excellent selection of procedure parameters and reagents (data not shown). No cross-reactivity was observed with any of the potential cross-reactants tested.

Establishing a reference standard serum encountered some difficulties. Contrary to previous studies (Miura et al., 2008; Dąbrowska et al., 2014) we worked only on a human model. We could not immunize healthy humans with phages for obvious reasons. No commercial kit for detection of the human antiphage antibodies is yet available on the market as well. Obtaining a sufficient amount of highly reactive patient's serum was limited due to a small volume of blood samples taken from patients and a relatively weak signal obtained in the ELISA test. From several dozen samples we chose two representatives; one was used for assessing IgG and IgM antibody levels, and the second one was used for assessing IgA levels. Dilutions of standard sera were made in twofold steps starting with 1:100 for IgA and IgM and 1:1000 for IgG to generate a four-point standard curve for each type of antibody. The AU were then calculated from their absorbance values at 450 nm using the parameters estimated from each standard curve (Miura et al., 2008). The average value of each duplicate (per sample) was calculated. The dilution giving an optical density at 450 nm of ≤1 was assigned as 1 000 AU (AUs). Achieving high values of absorbance (∼1 at 450 nm) for IgG caused no problems for us. However, due to weak reactivity of serum samples, we were able to obtain maximum values of absorbance at 0.6 for IgM and 0.25 for IgA, respectively. Reference sera as standards were run on each tested microplate.

Statistical Evaluation of the Data

The evaluation of statistical significance of differences between groups in the neutralization test was performed using Wilcoxon's test (for dependent variables) and the Mann–Whitney *U* test (for independent variables).

Results carried out by ELISA technique were analyzed using Wilcoxon's test (for dependent variables). For independent variables, we used the Mann–Whitney's *U* test and Student's *t*-test. To choose the appropriate statistical test we confirmed whether in groups of IgG, IgA, and IgM antibodies distributions were normal and whether there were homogeneous variances. In case of normal distribution and homogeneous variances we applied parametric Student's *t*-test. In remaining cases we used nonparametric Mann−Whitney's *U* test.

Significance was set at $p < 0.05$ and $p < 0.001$. Analysis was performed using the Statistica 10 software package.

RESULTS

As mentioned before, each patient subjected to experimental phage therapy was treated individually by a physician in charge according to the protocol of the experimental phage therapy employed at the Phage Therapy Unit. Depending on the overall health, the type of infection and its severity, a specific treatment was applied after determination of the sensitivity of isolated bacterial strains to the specific phage. Follow-up visits (during which blood samples were taken) took place at various time intervals in every single case. The number of follow-up visits varied from 1 to 11 depending on the course of the therapy. Sometimes the therapy was terminated by a physician (if eradication of infection was achieved or poor toleration occurred), while in other cases patients terminated therapy themselves due to the lack of expected improvement during treatment. Furthermore, the duration of the treatment varied from one month to over a year (with interruptions). The main criterion was patients' clinical condition confirmed by laboratory data. It should be emphasized that it is not unusual for patients with chronic bacterial infections (in contrast to acute infection episodes) to be treated with antibacterial agents for months (Leekha et al., 2011). Thus, it is hard to

summarize and standardize all collected data. Therefore, it was impossible in this experimental therapy to standardize sample collections similarly to protocols of clinical trials. Here, we decided to focus on serum samples obtained before therapy and on samples giving maximum levels of antiphage antibodies during the whole treatment (in the case of a single follow-up visit, we evaluated the one serum sample we had). These results were compared with the level of phage inactivation (*K* rate) measured in the neutralization test and with the overall outcome of phage treatment for each patient individually.

Effect of Phage Treatment on the Level of Antiphage Antibodies

We focused mainly on the immune response to the *S. aureus* MS-1 phage cocktail as phage preparations were applied exactly in such composition to all patients. Here, we present the level of anti-MS-1 phage IgG (AU) measured by the ELISA test for all 20 examined patients (**Figure 1A**). Values obtained individually for all 10 healthy donors who had never been subjected to phage therapy are shown in **Figure 1B**. The comparison of these two groups (mean values) are presented in **Figure 1C**. Due to the high diversity in the obtained results, the median values for the group of patients during treatment were evaluated (**Figure 1A**). The median level of IgG antibodies reached the highest values in comparison to the levels of IgA and IgM and was 78.95 AU. None of the healthy volunteers reached this limit. The mean value of the level of antiphage antibodies (IgG) for the group of patients before treatment was 61.71 AU and 166.62 AU during treatment. For healthy donors the mean value was 27.75 AU. Earlier bacterial infections and exposure to relevant phages were probably responsible for higher initial antibody values in patients compared with healthy individuals.

In contrast to the results described above, levels of antiphage IgA were the lowest in the group of patients as well as in the group of healthy donors (**Figures 2A,B**) in comparison to the levels of IgG and IgM. In fact, we did not detect any antiphage IgA in sera of healthy people. Similar results were obtained in patients (60% of examined patients

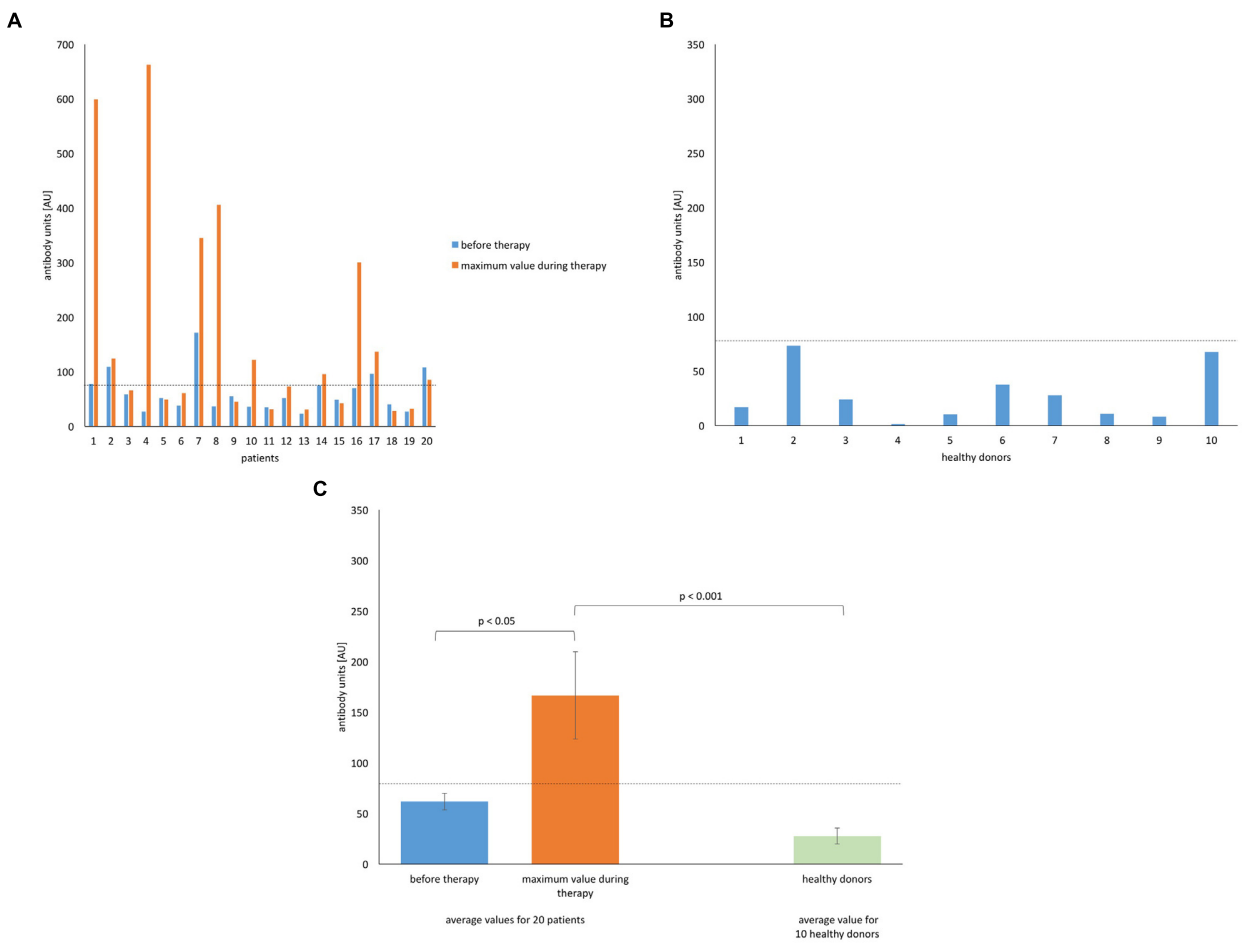

FIGURE 1 | Levels of IgG anti-MS-1 phage antibodies in human sera. (A) Patients ($n = 20$) treated with *S. aureus* MS-1 phage cocktail. **(B)** Healthy ($n = 10$) donors. **(C)** Average values for patients treated with *S. aureus* MS-1 phage cocktail and healthy donors. Average values are plotted with the standard error (SE) indicated by the error bars. Median value for the group of patients during treatment is marked on each graph by a horizontal dotted line.

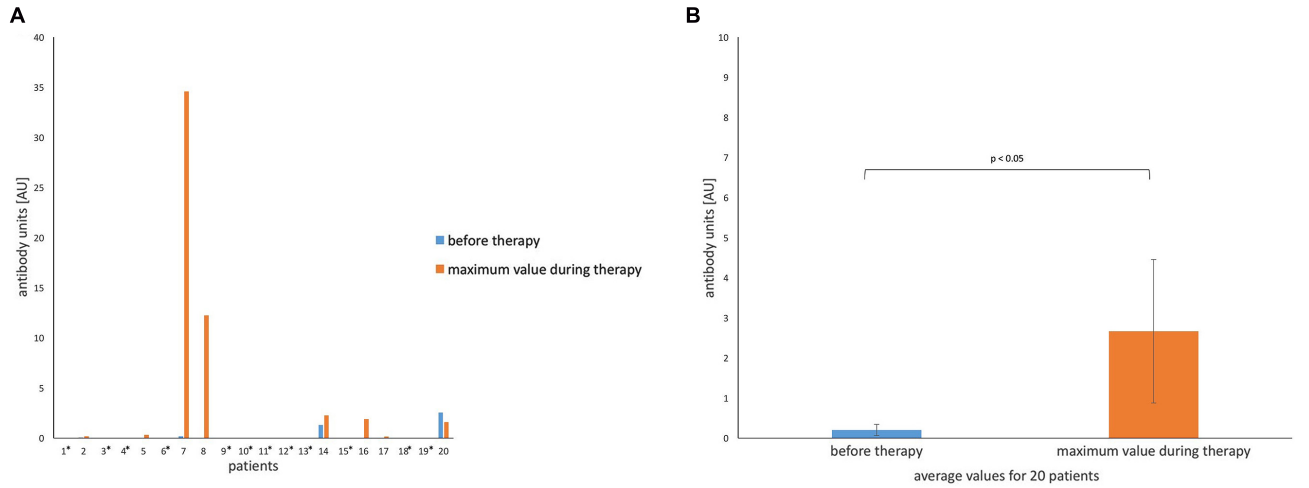

FIGURE 2 | Levels of IgA anti-MS-1 phage antibodies in human sera. (A) Patients ($n = 20$) treated with *S. aureus* MS-1 phage cocktail. An asterisk next to the number of a patient means undetectable levels of antibodies before and during treatment. **(B)** Average values for patients treated with *S. aureus* MS-1 phage cocktail before and during treatment. Average values are plotted with the SE indicated by the error bars. The average value for 10 healthy donors was zero. Median value for the group of patients during treatment was zero as well.

indicated undetectable levels of IgA before and during the whole treatment). This phenomenon occurred even in patients with sinus infections, where the mucosal immune system was stimulated by local administration of phages. Possibly, higher levels of IgA would be detected in saliva instead of serum. The median value of IgA obtained for the group of patients during treatment was zero as well. The mean value of the level of antiphage antibodies (IgA) for the group of patients before treatment was 0.2 AU and 2.66 AU during treatment.

The levels of anti-MS-1 phage IgM were the most surprising, as we observed an astonishingly high spread between the lowest and highest values in some patients (**Figure 3A**). Samples of two patients reached levels over 1 000 AU as a response to local phage administration (patients 7 and 17). In another two cases we did not detect any IgM elicited by phage before or during phage therapy (patients 13 and 15). Despite high levels of IgM in some cases, the median value in the group of patients during treatment was only 4.99 AU. The group of healthy subjects was characterized by relatively low levels of IgM antiphage antibodies, which were close to zero (**Figures 3B,C**). The mean value of the level of antiphage antibodies (IgM) for the group of patients before treatment was 11.84 AU and 205.89 AU during treatment. For healthy donors the mean value was 0.81 AU.

Three patients (patients 7, 8, and 16 in **Figures 1–3**) with markedly higher levels of all antibody isotypes (IgG, IgA, IgM) were treated by phages locally. However, in cases with low levels of IgG and IgM antiphage antibodies and with undetectable levels of IgA antiphage antibodies (numbers 13 and 19 in **Figures 1–3**) phages were applied likewise locally. These results are consistent with our earlier report, where no relationship between the route of administration and ELISA results was observed (Łusiak-Szelachowska et al., 2014). Statistical analysis of ELISA results in patients and healthy donors are summarized in **Tables 3** and **4**.

A long-term study of the four patients with the largest numbers of control visits showed meaningful differences in the levels of particular types of antiphage antibodies (**Figures 4A–D**). Apparently, IgA antibodies in sera were not involved in the humoral immune response during treatment as even long-term exposure (several months) to phage antigens did not induce secretion of IgA. In the case of patient 4 (**Figure 4A**), the *S. aureus* MS-1 phage cocktail was applied only for the first 76 days of therapy (*S. aureus* A5/80 and *S. aureus* φ200 phages were applied afterward). However, even a change of medication resulted in a noticeably stronger immune response by IgG antibodies to the MS-1 phage cocktail over a year after its administration. No changes in secretion of IgA antibodies were observed. In accordance with expectations, a marked increase of IgM was noted before the IgG boost directly after initiation of the treatment and after change of the phage preparation (from *S. aureus* MS-1 phage cocktail to *S. aureus* A5/80 phage). Analogously, even more rapid growth of IgM levels at the beginning of the treatment was noted in patient 8 (**Figure 4C**). Such dependence was not observed in another two cases (**Figures 4B,D**). IgG antibodies were mostly responsible for the humoral immune response in patients undergoing phage treatment. They persisted in the blood for several months during phage administration. All four patients analyzed in **Figure 4** received, at a certain stage of the treatment, another staphylococcal phage (different from the MS-1 phage cocktail). We did not evaluate the immune response to other phages used in treatment due to a lack of purified specific phage preparation.

Immunogenicity Depending on the Type of Phage

As stated above, beside investigations on the MS-1 phage cocktail, we measured likewise the immunological response to each phage

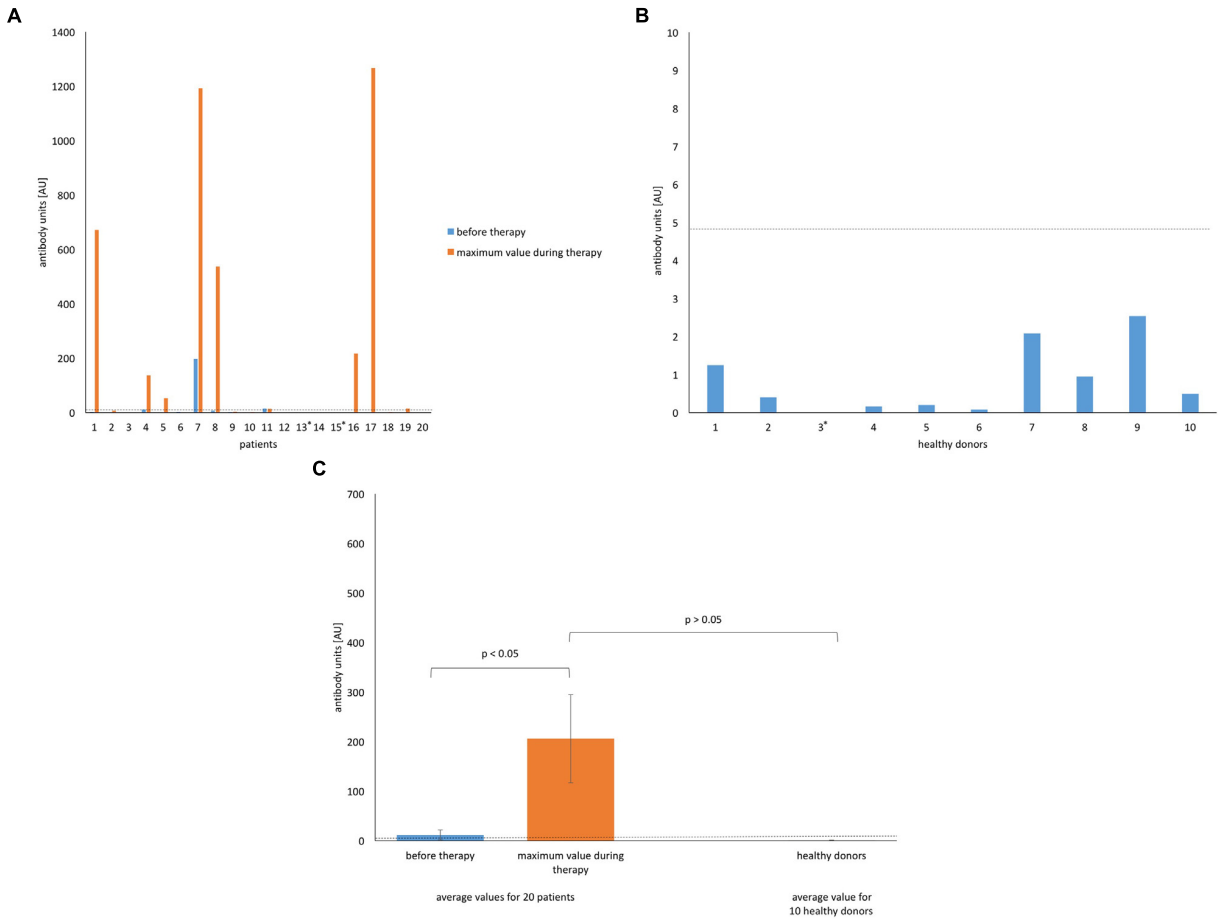

FIGURE 3 | The levels of IgM anti-MS-1 phage antibodies in human sera. (A) Patients ($n = 20$) treated with *S. aureus* MS-1 phage cocktail. **(B)** Healthy ($n = 10$) donors. An asterisk next to the number of a healthy donor means an undetectable level of antiphage antibodies. **(C)** Average values for patients treated with *S. aureus* MS-1 phage cocktail and healthy donors. Average values are plotted with the SE indicated by the error bars. Median value for the group of patients during treatment is marked on each graph by a horizontal dotted line.

TABLE 3 | Statistical analysis of ELISA results in 20 patients (response to MS-1 phage cocktail).

Type of antibody	Mean level of antiphage antibodies (AU) before therapy ± SE	Mean level of antiphage antibodies (AU) during therapy ± SE	Statistical significance (*p*)
IgG	61.71 ± 8.19	166.62 ± 43.06	0.008*
IgA	0.2 ± 0.14	2.66 ± 1.79	0.049*
IgM	11.84 ± 9.8	205.89 ± 88.71	0.002*

**Wilcoxon's test.*

from the cocktail separately. We chose two patients with a similar course of treatment. One of them showed a relatively high level of antiphage antibodies (especially IgA and IgM), while the second one revealed a much lower immune response. We noted that the response rate may depend not only on the type of applied phage but also on the type of antibody involved in binding to phage antigen. For IgG antiphage antibodies the most immunogenic (excluding the MS-1 phage cocktail) was *S. aureus* A5/80 phage, while for IgA it was *S. aureus* 676/Z phage (even if considering generally low values for IgA antiphage antibodies). We did not observe such coincidence for IgM antiphage antibodies. In patient with high levels of IgM antibodies the response to each phage reached a value over 500 AU after 20 days of the treatment. In the second case, practically no phage-induced production of IgM antibodies was observed (**Figures 5A,B**). Based on these results, we can assume that differences in the phages' immunogenicity are related to the various structure of phage proteins, which is consistent with previous reports describing animal models (Capparelli et al., 2007; Dąbrowska et al., 2014; Majewska et al., 2015).

Correlation between the Levels of Antiphage Antibodies and Antiphage Activity of Human Sera

Stimulation of neutralizing antibodies has been one of the arguments of opponents of phage therapy. We observed a correlation between increased level of antiphage antibodies

TABLE 4 | Statistical analysis of ELISA results in 20 patients compared to 10 healthy donors (response to MS-1 phage cocktail).

Type of antibody	Mean level of antiphage antibodies (AU) in healthy people ± SE	Mean level of antiphage antibodies (AU) in patients during therapy ± SE	Statistical significance (*p*)
IgG	27.75 ± 7.86	166.62 ± 43.06	<0.001^
IgA	0	2.66 ± 1.79	0.305#
IgM	0.81 ± 0.28	205.89 ± 88.71	0.067^

^*Mann–Whitney's U test;* #*Student's t-test.*

detected in the ELISA procedure and higher rate of phage inactivation (K) mostly for IgG and IgM antibodies. No correlation was observed for IgA antibodies. Determining which type of antibody (IgG or IgM) is characterized by the strongest neutralizing properties toward phages is difficult, as the obtained results for IgG and IgM coincide with each other in almost every case with $K > 18$. However, it should be mentioned that even a very high rate of phage inactivation ($K > 18$) does not necessarily mean an unfavorable outcome of phage treatment. All changes in the levels of antiphage antibodies (IgG, IgA, IgM) measured by the ELISA test in patients before and during treatment were statistically significant (**Table 3**) at $p < 0.05$. Changes in the rate of phage inactivation (K) observed before and during phage therapy were statistically significant as well (**Table 5**) at $p < 0.001$. Results regarding mean K rate in healthy donors compared to patients during treatment are presented in **Table 6**. Summary data related to the levels of antiphage antibodies, rate of phage inactivation and clinical results of phage therapy are presented in **Table 7**.

Rates of Inflammatory Markers in Patients Treated with Phages

We also analyzed our data in the context of the selected inflammatory markers (data not shown) in patients before and during treatment. The inflammatory markers in patients' blood were assessed by commercial laboratory methods. Two classical inflammatory markers, CRP (C-reactive protein) and

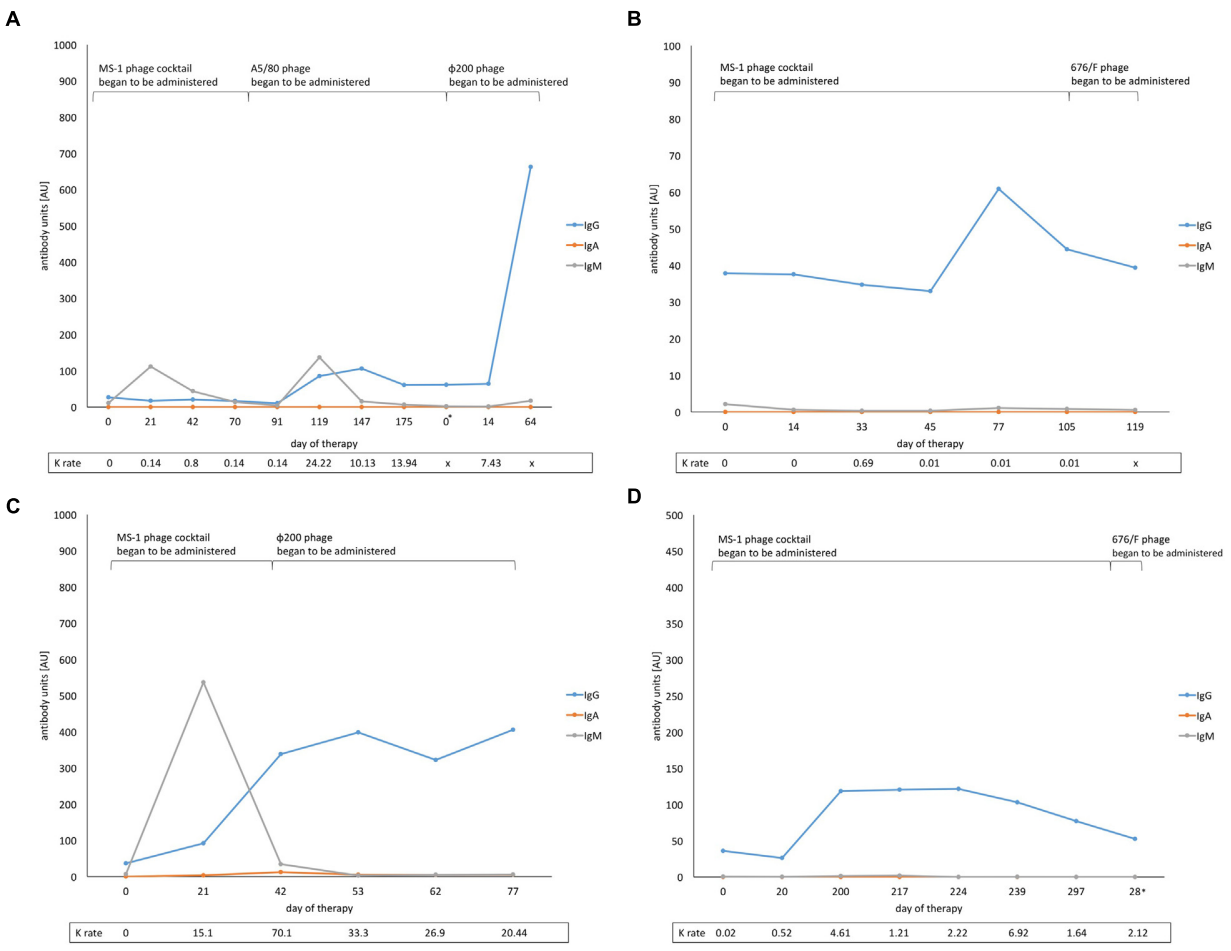

FIGURE 4 | Levels of anti-MS-1 phage antibodies (IgG, IgA, IgM) before and during phage therapy and antiphage activity of sera (AAS, *K* rate) in four patients with the largest number of control visits. (A) Patient 4; *means 5-month break between successive cycles of phage treatment, **(B)** Patient 6, **(C)** Patient 8, **(D)** Patient 10; *means 8-month break between successive cycles of phage treatment. *x, K* rate was not estimated.

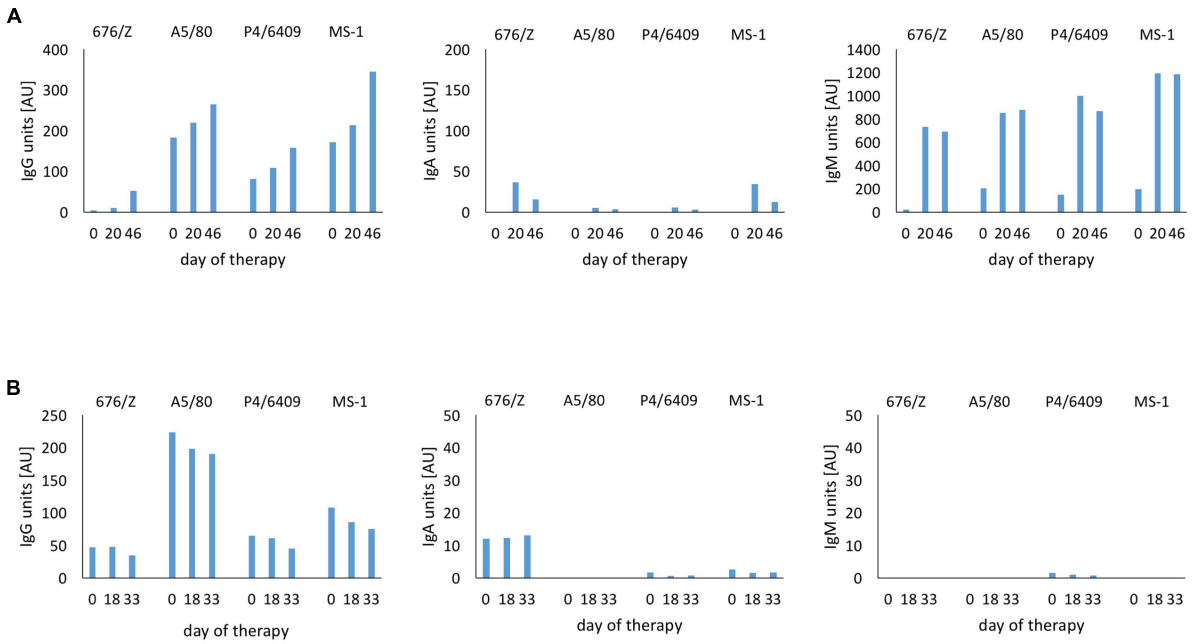

FIGURE 5 | Immunogenicity depending on the type of phage in patients treated with *S. aureus* MS-1 phage cocktail. (A) Patient 7. **(B)** Patient 20. The immunological response to each phage from the MS-1 phage cocktail was examined in the same way as phage cocktail. Purified single *S. aureus* phage preparations as antigens were diluted immediately before use to obtain the titer at 5×10^8 pfu/ml. The phage cocktail was prepared after diluting single phages in coating buffer by mixing equal volumes of three different *S. aureus* phage solutions (676/Z, A5/80, and P4/6409).

ESR (erythrocyte sedimentation rate) were considered. They are believed to be the most valuable markers for evaluating a patient's clinical status with respect to inflammation, infection, trauma or malignant disease (Saadeh, 1998; Międzybrodzki et al., 2009; Assasi et al., 2015). In the majority of cases both markers remained constant (with slight variations within the values) or were even lower during treatment than before phage therapy. In some cases, both markers decreased despite constantly increasing levels of antibodies (IgG and IgM) during treatment. Such data are partially consistent with previous results. Międzybrodzki et al. (2009) indicated a statistically significant reduction in mean CRP concentrations in patients during the first five weeks of phage treatment. Jończyk-Matysiak et al. (2015) observed that phage therapy had no effect on the level of inflammatory markers, ESR and CRP, when all data from patients observation were analyzed. Data are only partially consistent because the different patient cohorts, with various infections and undergoing different course of the treatment have been evaluated. It should be pointed out that purified phage preparations were applied in two cases (patient 1 was taking OP MS-1, whereas patient 8 was taking the OP MS-1 TOP phage preparation). Surprisingly, these two patients receiving purified phage preparations showed increased values of CRP during treatment (from 5.71 before therapy to 17.39 mg/l after two months of continuous treatment in patient 1 and from 5.9 to 8.52 mg/l after five weeks of treatment in patient 8). Furthermore, the purified phage preparations had no effect on lower induction of antibodies at all. In fact, patients treated with OP MS-1 and OP MS-1 TOP preparations had the highest levels of IgG and IgM antibodies measured by the ELISA technique within the whole tested group.

TABLE 5 | Statistical analysis of results from neutralization test in 20 patients (response to MS-1 phage cocktail).

Type of phage used in phage therapy	Mean *K* rate before therapy ±SE	Mean *K* rate in patients during therapy ±SE	Statistical significance (*p*)
S. aureus MS-1 phage cocktail	0.02 ± 0.01	23.78 ± 12.13	<0.001*

**Wilcoxon's test.*

TABLE 6 | Statistical analysis of results from neutralization test in 20 patients compared to 10 healthy donors (response to MS-1 phage cocktail).

Type of phage used in phage therapy	Mean *K* rate in healthy subjects ±SE	Mean *K* rate in patients during therapy ±SE	Statistical significance (*p*)
S. aureus MS-1 phage cocktail	0.003 ± 0.002	23.78 ± 12.13	<0.001^

^*Mann–Whitney's U test.*

DISCUSSION

The results from earlier reports are consistent with our current examinations and clearly show that bacteriophage can induce production of antiphage antibodies (mostly IgG and IgM) which

TABLE 7 | Immune response and clinical outcome of phage treatment in 20 examined patients.

Patient	IgG (AU) before therapy	IgG (AU) during therapy*	IgA (AU) before therapy	IgA (AU) during therapy*	IgM (AU) before therapy	IgM (AU) during therapy*	Phage inactivation (*K*) before therapy	Phage inactivation (*K*) during therapy*	Clinical outcome of phage therapy
1	77.82	598.84	0	0	2.07	671.4	0	204.49	F
2	108.91	124.04	0.06	0.17	0.21	6.56	0.01	0.14	F
3	58.44	65.74	0	0	0.07	0.09	0.02	0.13	E
4	26.8	662.56	0	0	10.99	136.68	0	13,94	E
5	51.72	48.87	0	0.34	0	52.82	0.03	10.84	F
6	37.82	60.92	0	0	2.15	1.12	0	0.69	E
7	171.45	345.1	0.19	34.59	197.35	1193.08	0.02	140.85	B
8	36.47	405.44	0	12.26	6.87	536.94	0	70.1	A
9	54.9	45.01	0	0	1.23	3.43	0	0.128	B
10	36	121.74	0	0	0.55	1.79	0.02	6.92	A
11	34.87	30.94	0	0	14.36	13.15	0.02	0.4	D
12	51.66	72.78	0	0	0.07	0	0	0.11	B
13	23.09	30.59	0	0	0	0	0.12	0.4	F
14	74.96	95.51	1.31	2.27	0	0.41	0.01	0.95	E
15	48.65	42.17	0	0	0	0	0.01	0.09	C, F[#]
16	69.92	300.28	0	1.9	0	216.57	0	10.26	D
17	95.96	136.56	0	0.16	0.61	1267.52	0.04	0.52	C
18	40.25	28.01	0	0	0.08	1.74	0.02	0.11	F
19	26.74	32.1	0	0	0	14.53	0.01	14.58	E
20	107.82	85.13	2.55	1.59	0.14	0.06	0	0.01	F

**Maximum values achieved during treatment; [#]C, chronic sinusitis; F, chronic conjunctivitis.*

may be responsible for phage inactivation. However, the immune response to phages depends on many different factors (duration of the treatment, phage dosage and route of administration). The immune status of a patient is no less significant. Previous reports indicated that several patients from our Phage Therapy Unit had immune deficits caused by infections, antibiotic treatment, etc. (Kurzępa-Skaradzińska et al., 2014). Górski et al. (2006) assumed that phage translocation in patients may be much higher than in healthy people as the gut barrier in disease is often much more permeable to microorganisms. Furthermore, in patients with immunodeficiencies, applied phages are believed to have longer viability (Borysowski and Górski, 2008). Some sources have noted that toxins produced by pathogenic bacteria in humans may have an inhibitory effect on their immune response (Gobert et al., 2007). Hentzer et al. (2001) found that temperate *P. aeruginosa* phages contribute to production of modified biofilm by pathogenic *Pseudomonas* strains, which is more resistant to antimicrobial treatments and activity of the immune system cells. As stated before, such data are still limited, especially when relating to human models. Pescovitz et al. (2011) noted that φX174 phage circulated for 3 to 4 days after intravenous application in healthy people until IgM antibodies completely inactivated phage particles before day 7. IgG antibodies were induced as well. In investigations by Bruttin and Brussow (2005), no humoral immune response was observed after oral administration. The authors suggested that no substantial amount of T4 phage ever appeared in their blood. Our group obtained similar results (Łusiak-Szelachowska et al., 2014). Although, we did not demonstrate the presence of phages in blood, such suggestion seems to be reasonable. Oral application induced the weakest immunological response, but, in this study, similar low levels of antibodies were observed in many patients after local administration as well. In our study we were unable to assess the direct relationship between the route of administration and the intensity of the immune response, as the majority of the tested group received phages locally or both locally and orally (only in two cases were phages applied entirely orally).

Some sources indicate that antiphage antibodies may be present in patients' sera before phage treatment or even in sera of completely healthy people. Kucharewicz-Krukowska and Ślopek (1987) observed the presence of specific antiphage antibodies in 23% of patients before phage treatment. Our earlier work (Łusiak-Szelachowska et al., 2014) indicated that a low level of antiphage activity of patients' sera could be detected before treatment. Dąbrowska et al. (2014) obtained congruous findings when investigating the level of anti-T4 phage IgG in the human population. Over 80% of healthy individuals were found to have antiphage antibodies in their sera. Those antibodies, so-called "natural antibodies" (Górski et al., 2012), may be a result of high prevalence of phages, which are well known for their vast abundance in almost every environment, even in the water supply system of European cities (Weber-Dąbrowska et al., 2014), and can induce antibody production in healthy controls. Our trials showed the presence of antiphage IgG antibodies in the

group of 10 healthy donors at similar levels as in the group of patients before implementation of phage therapy. We also found correlations between the levels of antiphage IgA (undetectable levels in the group of healthy people and exceptionally low values in patients before and at the time of the treatment). Differences were found in the levels of antiphage IgM. A few patients exhibited relatively high levels of antiphage IgM antibodies before phage application. Those levels increased rapidly at the beginning of the treatment.

The results obtained for two patients administered purified phage preparations provide interesting knowledge for further development of phage therapy. Generally, the patients subjected to treatment in the Phage Therapy Unit in Wrocław, Poland receive phage lysates (phage particles suspended in liquid bacterial medium) instead of purified preparations. Phage lysates may contain macromolecules derived from the host bacteria and culture medium. Their presence raises an important concern regarding the safety of the therapy (Szermer-Olearnik and Boratyński, 2015). Our preliminary results suggest that those concerns are unfounded. Based on the above observations, we can presume that application of phage lysates does not cause any deleterious effects compared to purified phage preparations. However, emphasis should be laid on phage titer in both phage preparations, which was four ranges lower in phage lysates, making them possibly less immunogenic. It appears that phage dose, not the level of purification of phage solution, plays the most important role in immunogenicity of therapeutic phage preparations in humans. However, larger cohorts of patients are needed to draw definite conclusions.

Data from animal models are more numerous. Results obtained by Stashak et al. (1970) and Sulakvelidze and Barrow (2005) indicated the appearance of antiphage antibodies in animals which are able to inactivate phages during treatment. The latest reports have presented similar results. In a murine model, specific immunization to T4 head proteins decreased phage activity *in vitro* and *in vivo* in a group pre-immunized with phage Hoc protein but only *in vitro* in a group pre-immunized with gp23 protein (Dąbrowska et al., 2014). Differences in the immunogenicity of phage structural proteins were reported by other authors. Capparelli et al. (2007) described two staphylococcal phages, wild type and its mutant driven by the mice immune system, which were serologically distinct. The mutant was persistent in neutralizing antibodies in the mouse circulation, whereas the wild phage was almost completely inactivated within 2 days after intravenous application. A long-term study of antibody induction in mice by T4 phage applied orally in very high doses (4×10^9 pfu/ml thus making approximately 2×10^{10} pfu/mouse daily; mice were fed with T4 phage in drinking water for 100 days) showed a significant increase in antibody levels (IgG in sera after 36 days of treatment and IgA in feces after two months of continuous treatment). The increased IgA level antagonized gut transit of active phage (Majewska et al., 2015).

Finding similarities among animal and human models encounters difficulties due to certain limitations of this study. No doubt, animal experiments can be planned more accurately. We were not able to assess phage viability in human tissues as was done in animal investigations (Hodyra-Stefaniak et al., 2015) or in the gut. The results gained so far from human stool samples (Łusiak-Szelachowska et al., 2008) did not reflect the full scope of events taking place within the gut during phage treatment. Similar obstacles were faced by Sarker et al. (2016). Considering the immunogenicity of phages in the animal gut, the term "rather low" has been used (Majewska et al., 2015). However, one cannot directly extrapolate those studies in mice to the clinic.

CONCLUSION

Overall, the majority of studies to date show that phages may induce a humoral immune response in humans. Interpretations regarding the strength of this response are often contradictory or unclear. As described above, patients who showed the highest level of antiphage antibodies and the highest antiphage activity of their sera ended phage treatment with good clinical results or even with full recovery. The significance of these findings is not to be underestimated, as the results from animal experiments cannot be simply transferred to a human model. Our studies confirm and extend our earlier work indicating that phage therapy may induce various levels of antibody formation which does not necessarily affect the outcome of therapy. Evidently, further studies are needed to shed more light on phage-dependent immune responses and their significance for the success or failure of therapy.

AUTHOR CONTRIBUTIONS

MŻ, MŁ-S, EJ-M, BW-D, BO, AK, AG: designed the experiments, analyzed the data, wrote and revised the manuscript, approved the version to be submitted. RM, WF, PR: collected and analyzed data from patients, revised the manuscript, approved the version to be submitted. RM, WF, PR, AG: conducted phage treatment in patients. MŻ, MŁ-S, BO, AK: performed the experiments.

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'Get in Early'; Biofilm and Wax Moth (*Galleria mellonella*) Models Reveal New Insights into the Therapeutic Potential of *Clostridium difficile* Bacteriophages

*Janet Y. Nale[1], Mahananda Chutia[2], Philippa Carr[1], Peter T. Hickenbotham[1] and Martha R. J. Clokie[1]**

[1] Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK, [2] Pathology and Microbiology Division, Central Muga Eri Research and Training Institute, Assam, India

Clostridium difficile infection (CDI) is a global health threat associated with high rates of morbidity and mortality. Conventional antibiotic CDI therapy can result in treatment failure and recurrent infection. *C. difficile* produces biofilms which contribute to its virulence and impair antimicrobial activity. Some bacteriophages (phages) can penetrate biofilms and thus could be developed to either replace or supplement antibiotics. Here, we determined the impact of a previously optimized 4-phage cocktail on *C. difficile* ribotype 014/020 biofilms, and additionally as adjunct to vancomycin treatment in *Galleria mellonella* larva CDI model. The phages were applied before or after biofilm establishment *in vitro*, and the impact was analyzed according to turbidity, viability counts and topography as observed using scanning electron and confocal microscopy. The infectivity profiles and efficacies of orally administered phages and/or vancomycin were ascertained by monitoring colonization levels and larval survival rates. Phages prevented biofilm formation, and penetrated established biofilms. A single phage application reduced colonization causing extended longevity in the remedial treatment and prevented disease in the prophylaxis group. Multiple phage doses significantly improved the larval remedial regimen, and this treatment is comparable to vancomycin and the combined treatments. Taken together, our data suggest that the phages significantly reduce *C. difficile* biofilms, and prevent colonization in the *G. mellonella* model when used alone or in combination with vancomycin. The phages appear to be highly promising therapeutics in the targeted eradication of CDI and the use of these models has revealed that prophylactic use could be a propitious therapeutic option.

Keywords: *Clostridium difficile*, *Clostridium difficile* infection, biofilms, *Galleria mellonella*, bacteriophages, bacteriophage therapy

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****Correspondence:***
Martha R. J. Clokie
mrjc1@le.ac.uk

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INTRODUCTION

Clostridium difficile is an important human and animal pathogen and a major cause of pseudomembranous colitis where it accounts for 15–39% of antibiotic-stimulated toxin-mediated diarrhea (McFarland, 2009; Viswanathan et al., 2010). *C. difficile* infections (CDI) are becoming increasingly severe due to limited treatment options and the emergence of pathogenic ribotypes

(Freeman et al., 2010; Zucca et al., 2013). Complications of the disease can also arise from antibiotic resistance, leading to relapse, increased health care-associated costs and death in 10% cases (Wiegand et al., 2012; Lessa et al., 2015; Vindigni and Surawicz, 2015). Therefore, there is a need for specific and efficient strategies for the targeted eradication of this pathogen.

The pathogenicity of *C. difficile* is linked to potent cytotoxins (toxin A, B and binary toxin AB) which together are responsible for the damage of the epithelial lining leading to pseudomembranous colitis (Lyerly et al., 1988; Carter et al., 2007; Lyras et al., 2009). Other factors such as fimbriae and surface layer proteins contribute to its motility and adhesion to the ileum and caecum where the disease is most prominent (Borriello et al., 1988; Tasteyre et al., 2000). In addition, their hardy spores resist heat and desiccation, thus playing a significant role in their spread, survival and disease (Akerlund et al., 2008; Burns et al., 2011). Of particular relevance to this paper, *C. difficile* also produces biofilms, which consist of aggregates of cells embedded in self-produced extracellular polymetric substance (EPS) (Branda et al., 2005; Dawson et al., 2012; Đapa et al., 2013). The EPS matrix binds the spores and vegetative cells, and provides protection for the bacteria against oxygen stress and enhances their adhesion to abiotic surfaces (Dawson et al., 2012). Therefore, biofilms contribute to *C. difficile* virulence by potentially enhancing persistence and proliferation of the pathogen in the environment, and during active infection where they could interfere with the activity of antimicrobial agents and treatment of the disease.

The conventional CDI treatment relies solely on three antibiotics: metronidazole, vancomycin and fidaximicin, although limitations to their use have been reported (Aslam et al., 2005; Pepin, 2008; Crawford et al., 2012). Metronidazole is not effective for the treatment of all ribotypes, vancomycin is predominantly used as a treatment of last resort due to the possibility of resistance emergence. Fidaximicin is much newer and is not cost effective as a first-line treatment for some strain-specific CDIs (Wiegand et al., 2012; Bartsch et al., 2013; Chilton et al., 2014). In terms of mechanisms of action, metronidazole disrupts DNA replication, vancomycin targets the bacterial cell wall and fidaximicin hinders RNA polymerase activity (Watanakunakorn, 1984; Löfmark et al., 2010; Venugopal and Johnson, 2012). Clearly antibiotic action is hampered by the limited access to the *C. difficile* due to obstacles posed by the hardy spores and biofilm formation. Pertinent to the biofilm is the EPS matrix which could be overcome by antimicrobial agents such as bacteriophages (phages) (Parasion et al., 2014; Chan and Abedon, 2015; Abedon, 2016).

Phages are viruses that specifically infect bacteria. By infecting and then lysing biofilm-causing bacteria they have been shown to prevent biofilm formation (Parasion et al., 2014; Chan and Abedon, 2015; Dalmasso et al., 2015; Abedon, 2016). In addition, the EPS-depolymerases carried by some phages, which have a different function than lysins (lysis of the bacterial cell), impact biofilms by degrading EPS matrix (Parasion et al., 2014; Chan and Abedon, 2015). This enzyme activity presumably exposes the bacterial cells to antimicrobial agents, and provides access for phages to the receptors found on the cell wall, leading to infection and lysis. These advantages of phages over antibiotics is attractive and could be exploited either for phage mediated eradication of *C. difficile*, or to enhance antibiotic activity.

Phages have been developed for therapeutic purposes to treat bacterial infections caused by *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Proteus,* and *Escherichia coli* (Slopek et al., 1987; Abedon et al., 2011; Brüssow, 2012). In addition to medical uses, their safety, specificity and ability to replicate *in situ* has meant that phages both can and are currently used in the food industry as alternatives to antibiotics and as decontamination agents (EBI Food Safety, 2007; Hagens and Loessner, 2010; Kumari et al., 2011). Although several *C. difficile* phages have been characterized, few studies have focused on their potential applications for the treatment of CDI (Zucca et al., 2013; Hargreaves and Clokie, 2014; Nale et al., 2016). Most therapeutic studies have investigated single phages, and reported the isolation of resistant and lysogenic clones (Ramesh et al., 1999; Govind et al., 2009; Meader et al., 2010, 2013; Nale et al., 2016). In contrast, our previous work demonstrated that the use of optimized phage combinations could mitigate lysogeny and resistance *in vitro*, and could reduce colonization and extend the life expectancy of animals in a hamster model of CDI (Nale et al., 2016).

The other previous work on therapeutic CDI phages has concentrated on exploiting an artificial human gut model (Meader et al., 2010, 2013), and on the hamster model (Ramesh et al., 1999; Govind et al., 2011; Nale et al., 2016). Both are useful but have limitations. The artificial human gut model has been used to reveal many facets of enteric pathogens but is has a large footprint and the experiments are technically difficult to run. Although the hamster CDI model is good due to the ability of the animals to demonstrate various clinical symptoms of the disease that are comparable to humans, these animals are susceptible to the toxins, and succumb to the disease easily (Price et al., 1979; Best et al., 2012). The technical limitations of these models, the lengthy process for ethical/licenses approvals, and limitations associated with cost and space are causing researchers to shift interest toward exploring other substitutes for *C. difficile* research. Recent studies, for example, have examined zebra fish embryos to test *C. difficile* toxin B clones (Hamm et al., 2006; Lanis et al., 2010).

Galleria mellonella larvae have been reported to be a suitable alternative model to larger mammals for bacterial colonization studies (Ramarao et al., 2012) and an excellent tool for pharmacokinetics studies of antimicrobials (Hill et al., 2014). The model has also been used to study pathogenesis of bacteria such as *Pseudomonas aeruginosa*, *Listeria monocytogenes, Bacillus cereus,* and *Francisella tularensis* (Miyata et al., 2003; Aperis et al., 2007; Fedhila et al., 2010; Mukherjee et al., 2010) and phage therapy for *Burkholderia cepacia* and *P. aeruginosa* (Seed and Dennis, 2009; Beeton et al., 2015). However, no studies have been published to date to examine how useful *Galleria* would be as a model for CDI colonization, or subsequent phage therapeutic studies. There have been limited studies on the optimal dosing of phages and it is likely that significant amounts of knowledge can be gained from this model to inform subsequent animal experiments.

In this study, we examined the ability of *C. difficile* phages to target this bacterium in two models of infection. We investigate the efficacy of an optimized phage cocktail to prevent or treat *C. difficile* biofilms, and as a stand-alone treatment or combined with vancomycin in the *G. mellonella* larvae CDI model. We show that the phages prevent biofilm formation *in vitro*, and penetrate into and reduce established biofilms. In the *Galleria* model, the phages are most effective when used prophylactically, and in the presence of vancomycin, with multiple doses required to produce comparable results in the remedial regimen. It is clear that phages are able to impact *C. difficile* biofilms and significantly reduce the extent of colonization in a *G. mellonella* model of CDI and therefore appear to have great potential for the treatment of this bacterial pathogen. These models can be used to pursue aspects of phage therapy development in the future, such as to develop dosing and timing regimens.

MATERIALS AND METHODS

In vitro Phage Treatment of Biofilms

Bacterial Isolates and Phages

Six *C. difficile* ribotype 014/020 human clinical strains (CD105LC2, ATJ, AIP, ATK, TL176 and AUS1022) were examined in this study (Nale et al., 2016). The biofilm characteristics of R20291 were previously established and therefore was used as a control strain in the biofilm preliminary assays (Dawson et al., 2012; Đapa et al., 2013). The four phages examined, CDHM1, 2, 5, and 6 were previously isolated from our laboratory and propagated individually on the environmental *C. difficile* strain, CD105HE1 (Hargreaves et al., 2014; Nale et al., 2016). The filtered phage lysates (using 0.22 μl filters, Merck Millipore Ltd, Cork, Ireland) were mixed equally to form the cocktail. All the bacterial strains and phages tested were preserved in 25% glycerol stocks at −80°C.

Establishment of Biofilm Characteristics of the Bacterial Strains

Prior to phage treatment, the biofilm characteristics of the test strain CD105LC2 was first established *in vitro* on 12-well plates (Greiner Bio-One Ltd, UK) and the feature compared with the other five ribotype 014/020 strains using methods previously described (Đapa et al., 2013). Briefly, 48 h *C. difficile* cultures were produced on brain heart infusion (BHI) (Oxoid, UK) agar plates supplemented with 7% defibrinated horse blood (TCS Biosciences Ltd, UK). Broth cultures were prepared by inoculating a bacterial colony into 5 ml brain heart infusion supplemented (BHIS) with 0.1% w/v each of Ly-cysteine (Sigma–Aldrich, UK) and 5 mg/ml yeast extract (Oxoid, UK) and incubated at 37°C anaerobically for 18–24 h. Afterward, 1:10 dilutions of the overnight cultures (10^7 CFU/ml) were prepared in pre-reduced BHIS broth and 3 ml aliquots were added to the 12-well plates and incubated for 1–5 days. After each day the planktonic and biofilm vegetative cells and heat-resistant (at 65°C for 3 h) spores were enumerated on BHI agar and Brazier's cefoxitin, cycloserine and egg yolk (CCEY) agar plates (BioConnections, UK) respectively. To determine the biofilm mass, planktonic cultures from the wells were carefully removed to expose the thin biofilm layer at the bottom. The wells were washed twice with sterile phosphate buffered saline (PBS) and stained with 2 ml 0.1% filtered crystal violet followed by incubation at room temperature for 1 h. After washing off the crystal violet three times with PBS, 1 ml of absolute methanol was applied and incubated at room temperature for 5 min to extract the biofilm-bound stain. Approximately 250 μl was transferred to a 96-well plate and absorbance was read at 595 nm (Dawson et al., 2012; Đapa et al., 2013).

Phage Treatment of Biofilms

Having established the biofilm characteristics of the test strain, CD105LC2, a fresh biofilm of this strain was produced from a dilution of the overnight culture (Set 1) and subjected to four different phage treatment regimens (Sets 2–5, **Table 1**) using 300 μl of the single or phage combination (at 10^9 PFU/ml, MOI = 10). Sets 2, 3, and 4 represent biofilm pre-treatment regimens where phages were added: 1 h prior to bacterial exposure (Set 2), 1 h post-bacterial inoculation (Set 3) or simultaneously with the bacteria (Set 4). The only post- treatment regimen was Set 5. In this treatment, maximum biofilms (at 24 h) were established first and planktonic cultures removed as described above before treating them with the phages as shown in **Table 1**. The treated biofilms were further incubated for 24 h. After each treatment, the resultant vegetative cells and spores

TABLE 1 | Experimental set-up for the pre- and post-biofilm regimens with single and combined phages.

Regimen	Biofilm phage treatment regimens	Treatment
Set 1	Control (Untreated biofilms)	3 ml of 10% dilution of ON bacterial culture in pre-reduced BHIS was added to the wells.
Set 2	Pre-treatment 1	3 ml of the 10% diluted culture was added to the wells and incubated for 1 h. Afterward, 300 μl phage lysate/cocktail was added.
Set 3	Pre-treatment 2	300 μl of phage lysate/cocktail was added to the wells and incubated for 1 h. After incubation, 3 ml of 10 % diluted culture was added.
Set 4	Pre-treatment 3	3 ml of 10% diluted bacterial culture was mixed with 300 μl of phages and added to the wells.
Set 5	Post-treatment	3 ml of 10 % diluted bacterial culture was added to the plates and incubated overnight. Afterward, the planktonic cells were washed out and 300 μl of phage lysate/cocktail was added.

Phages were propagated individually (10^9PFU) and mixed together in equal proportions to constitute the cocktail. Biofilm treatment was conducted with 300 μl of the phage cocktail and 3 ml of the 10% diluted overnight bacterial cultures (MOI = 10) or on established biofilms of a C. difficile 014/020 isolate (CD105LC2). All treated biofilms were further incubated for 24 h before enumeration of the vegetative cells and spores.

were enumerated and the biofilm mass determined as described above.

Phage Treatment of Colony Biofilms

To determine the effect of phage treatment on the topology of biofilms, colony biofilms were produced on membrane filter disks (Merck Millipore Ltd, Ireland) and treated with the phage cocktail. To do this, the filter disks were sterilized both sides using Stratalinker UV crosslinker 2400 (Strategene, US) at 9999 μm setting. The sterilized disks were aseptically transferred onto BHI agar plates and 5 μl of a 10-fold dilution of the overnight bacterial culture was applied onto the filter disks. After 24 h incubation, a set of membrane disks for each day was transferred onto a fresh BHI agar plate and this was repeated up to 5 days. For phage treatments, 50 μl of the phage cocktail was gently applied to cover the entire surface of the biofilm after transferring biofilms to fresh medium each day. The treated biofilms were further incubated for an additional 24 h. Biofilms for each day (phage-treated and control) were removed and transferred to a tube containing 1 ml of cold BHI and vortexed (3000 rpm/min for 30 s) to dislodge the biofilm from the membranes. The membranes were removed and the resultant cultures were washed three times in ice-cold BHI and centrifuging at 15 000 *g* for 5. The vegetative cells and spores were enumerated using methods described above.

Scanning Electron and Confocal Microscopy

To conduct scanning electron microscopy (SEM) analysis on the biofilms, a set of phage-treated and control biofilms on the membrane disks were transferred to 12-well plates and sterilized with 1 ml of 2.5% gluteraldehyde (v/v in PBS) for at least 24 h. The membranes were processed through several washes in distilled, de-ionized water, followed by 30-min dehydration steps through a 30, 50, 70, 90, and 100% ethanol series. After two more 20-min washes through 100% analytical grade ethanol, the membranes were gradually infiltrated with Hexamethyldisilazane (HMDS) by 1 h washes in 2:1, 1:1 and finally 1:2 mixtures of ethanol:HMDS, followed by two 30-min washes in 100% HMDS. The HMDS was removed, and membranes were air dried overnight. Dried membranes were mounted onto 12.5 mm aluminum stubs and sputter coated with gold/palladium using a Quorum Q150T ES coating unit. Samples were observed, and the images recorded using Hitachi S3000H scanning electron microscope with an accelerating voltage of 10 kV.

For the confocal analysis, a different set of phage-treated and untreated colony biofilms were prepared on membrane filters as described and stained using FilmTracer Live/Dead biofilm Viability kit according to the manufacturer's recommendations (ThermoFisher Scientific, UK). Membranes were sterilized with the glutaraldehyde for 24 h. Confocal fluorescence imaging was conducted as previously described (Osman et al., 2016).

In vivo Analysis of Phage Therapy Using the *Galleria mellonella* Model

Preparation of Bacterial Inoculum

A dilution of the overnight inoculum of the bacteria was prepared as above and incubated until OD_{550} of 0.2 was attained. The culture was then centrifuged at 15 000 *g* for 5 min and the resultant pellet was re-suspended in cold BHI to a final concentration of 1×10^7 CFU/mL.

Bacterial Infection, Colonization and Treatment of *G. mellonella* Using a Single Phage Dose

Larvae of *G. mellonella* were obtained from Live Food UK Ltd. (Rooks Bridge, UK). On arrival, the larvae were stored immediately at 4°C and used within 1 week. Larvae with approximate weight of 0.25–0.30 g were selected for *in vivo* analysis (Abbasifar et al., 2014). The larvae were surface-sterilized with cotton swaps dipped in 70% ethanol. A single dose (in 10 μl) of either 10^5 CFU of bacterial inoculum, 10^6 PFU of phage cocktail or BHI broth was administered via the oral route using a 10-μl Hamilton syringe pump. Four randomly selected larvae were examined in each treatment regimen at 37°C in plastic Petri dishes. The insects remained unfed throughout the experiment (Ramarao et al., 2012). Two control groups were observed: the first received only bacteria (Control CD105LC2) and the second a dose of the phage cocktail (Control Phage). The treatment models were divided into either prophylactic or remedial groups. In the prophylactic model, phages were administered 2 h before bacterial infection (Phages + CD105LC2) or given simultaneously with the bacteria (Phages + CD105LC2 (s)). In the remedial group, bacteria were administered 2 h before the phage treatment (CD105LC2 + Phages).

Insects were considered dead when they become inert and turned black in color (Ramarao et al., 2012). Experiments were repeated three times. Survival curves were plotted using the Kaplan-Meier method in GraphPad Prism 6, and differences in survival rates were calculated by using the Log-rank (Mantel-Cox) test.

Bacterial and Phage Recovery from the Larvae

At the end of the experiment, the larvae were dissected dorsoventrally, the heamolymph extracted into ice-cold BHI, and phage and bacteria enumerated as described above. *C. difficile* colonies were confirmed with colony PCR targeting the *C. difficile* 16S rRNA (Rinttilä et al., 2004). For recovery of phages, homogenized heamolymph mixtures were centrifuged at 5000 *g* for 10 min at 4°C and the filtered supernatants were assayed for phages using spot test on the phage propagating host, CD105HE1, as the indicator strain. Recovery of phages from the feces was conducted by combining and suspending excreta from all four larvae in a group into 1 ml of cold BHI and incubated at 4°C for 1 h. The centrifuged and filtered supernatants were enumerated for phage presence as above. Data were analyzed using GraphPad Prism 6. All *in vivo* experiments were performed at least thrice.

G. mellonella DNA Extraction and qPCR

Heamolymph from the four larvae from the treatment groups above were combined and DNA was extracted from them using DNeasy Blood & Tissue Kit (Qiagen, Germany). Approximately, 50 ng of DNA from each group of larvae was subjected to RT-PCR targeting the *C. difficile* 16S rRNA using 7500 Fast Real Time PCR system with Fast SYBR Green Master mix (Thermo Fisher

Scientific, USA). Data was compared with standard CFU/ml counts in the growth curve of the test strain and analyzed using GraphPad Prism 6.

Treatment of *G. mellonella* Using Multiple Phage Doses

Since the above phage treatment was conducted using a single dose of the phage cocktail, further experiments were conducted to determine if multiple phage doses could improve the treatment regimen. To do this, five groups of larvae were given a mixture of phages/bacteria at the 0 h. This is the only treatment in group A, but in group B larvae subsequently received an additional single dose of phage cocktail at 6 h while group C received 2 doses at 6 and 12 h (**Table 2**). Group D larvae received three doses of phages at 6, 12 and 24 h while group E received 4 doses at 6, 12, 24, and 48 h (**Table 2**). Survival rates were recorded every 12 h for 72 h.

Phage/Antibiotic Combined Treatment on the Larvae

After phage therapy was established on the larvae, the phages were tested as adjunct to vancomycin, a commonly used antibiotic for CDI. Prior to the combined treatment, the windows of opportunity during which phages may be most effective as prophylactic therapeutics was determined by administering the phages to the larvae first. Time delays of 2 h (A, Phage prophylactic 1), 4 h (B, Phage prophylactic 2), 6 h (C, Phage prophylactic 3) or 12 h (D, Phage prophylactic 4) were observed before exposing them to bacterial infection (**Table 3**). To determine if the phage prophylaxis would aid vancomycin treatment, insects were given phages and in 2 h intervals challenged with bacteria and given vancomycin (64 μg/g, MIC $\geq$ 1 μg/ml) (E, Phage prophylaxis 5). Similarly, the ability of vancomycin prophylaxis to enhance phage therapy was also tested in treatment F (vancomycin prophylaxis) where vancomycin was given first and subsequently bacteria and phages were given at 2 h intervals respectively (Desbois and Coote, 2011). The treatments were compared with vancomycin treatment, (G, vancomycin given 2 h after exposure to bacteria) or vancomycin/phage pre-treatments, (H, vancomycin and phages were given simultaneously before exposure to bacteria 2 h later) or phage/vancomycin remedial regimen (I, vancomycin/phage simultaneously given 2 h post bacterial challenge) (**Table 3**).

Because CDI takes advantage of dysbiosis in the gut, another group of larvae were first treated with clindamycin (30 μg/g, MIC $\geq$ 1.125 μg/ml) to suppress bacterial commensals in the haemolymph. Subsequently, after 24 h the larvae were exposed to either bacteria followed by a dose of phage cocktail (J, Clindamycin pre-treatment 1) or given phage first before the bacteria (K, Clindamycin pre-treatment 2). All treatment groups were compared to the control bacterial group (L, which received just bacteria) (**Table 3**).

Larvae were culled at the 60th hour except for groups J and K, which required additional 12 h after the last exposure (**Table 3**).

TABLE 2 | Multiple phage dose regimens observed in the remedial treatments on *G. mellonella* larvae model of CDI.

Time to phage dose (h)	Phage dose regimens					
	Control	A	B	C	D	E
0	B	P, B	P, B	P, B	P, B	P, B
2			P	P	P	P
6				P	P	P
24					P	P
48						P
72	Cull	Cull	Cull	Cull	Cull	Cull

Larvae were inoculated with a mixture of phages (P) (10^6PFU) and/or bacteria (B) (10^5 CFU) in 10 μl volume and subsequently treated with additional doses of phages at the indicated time points. At the end of the experiment larvae were killed (culled) by placing them in −20°C for 5 min.

RESULTS

C. difficile Strains Have Different Biofilm Characteristics

To determine the characteristics of the test strain, CD105LC2, prior to phage treatment, we first established its ability to produce biofilms and compared this feature to other ribotype 014/020 strains and to the reference strain, R20291 (ribotype 027). In preliminary biofilm assays, absorbance readings showed that maximum biofilm was produced at 24 h. Therefore, all subsequent analyses were conducted on biofilms formed at this time. The ribotype 014/020 strains produced different levels of biofilm (Supplementary Figure S1A). ATJ, AIP, AUS1022 and TL176 were similar at OD_{595} ∼1.1, while the other two strains, ATK and CD105LC2 had readings of ∼1. 5. The biofilm produced by R20291 strain (OD_{595}∼1.7) at 24 h is comparable to previously published data (Dawson et al., 2012; Đapa et al., 2013). Analysis of viability counts also suggests that variable quantities of spores/vegetative cells are produced by the ribotype 014/020 strains both in the biofilms as well as in the planktonic cultures (Supplementary Figure S1B). Generally, there were higher spore counts in the planktonic cultures (10^5–10^7 CFU/ml) than in the biofilms (10^2–10^4 CFU/ml) but higher levels of vegetative cells in the biofilms (10^6–10^8 CFU/ml) compared to the planktonic cultures (10^2–10^4 CFU/ml) (Supplementary Figure S1B).

Phage Treatment Regimens on *C. difficile* Biofilms

Having established the biofilm characteristics of the test strain, we determined the impact of phages on biofilm formation. We examined the activity of the individual and combined phages when applied before or after the biofilm establishment, compared to the untreated biofilm (Set 1, **Figure 1A**). In all of the pre-treatment regimens examined (Sets 2–4), vegetative cells were completely eliminated in the planktonic cultures when treated with the phage combination (**Figures 1B–E**). When bacterial growth was established for an hour before phage treatment (Set 2), vegetative cells were eliminated but ∼10 and ∼ 10^2 CFU/ml of spores and vegetative cells, respectively, were detected in the biofilms (**Figure 1B**). Interestingly, when the phages were added first (Set 3), biofilm establishment was prevented and no bacteria were detected either in the biofilm

TABLE 3 | Phage prophylaxis regimens and phage/vancomycin combined treatment in *G. mellonella* larvae.

Time (h) → Treatments↓	0	2	4	6	12	24	25	60	72
A, Phage prophylaxis 1	P	B						Cull	
B, Phage prophylaxis 2	P			B				Cull	
C, Phage prophylaxis 3	P				B			Cull	
D, Phage prophylaxis 4	P					B		Cull	
E, Phage prophylaxis 5	P	B	V					Cull	
F, Vancomycin prophylaxis 1	V	B	P					Cull	
G, Vancomycin treatment	B	V						Cull	
H, Vancomycin/phage prophylaxis	VP	B						Cull	
I, Vancomycin/phage remedial regimen	B	VP						Cull	
J, Clindamycin pre-treatment 1	C					B	P		Cull
K, Clindamycin pre-treatment 2	C					P	B		Cull
L, Control bacteria	B							Cull	

The larvae were inoculated with 10 μl of phages (P), vancomycin (V) and/or clindamycin (C) at the indicated times. Bacterial enumeration from each larva was conducted from inactivated (culled) larva.

or planktonic cultures (**Figure 1C**). When bacteria and phages were added simultaneously (Set 4), $\sim 10^3$ CFU/ml of spores were recorded in the planktonic culture (**Figure 1D**). In the post-biofilm regimen (Set 5), phages were less effective and $\sim 10^2$ and 10^4 CFU/ml vegetative cells and spores were recovered respectively (**Figure 1E**). In contrast to treatments with the phage combination, the single phage treatments were less effective (**Figures 1B–E**).

Bacterial Enumeration and Microscopy Analysis Confirmed That Phages Can Penetrate *C. difficile* Biofilms

To further investigate the effect of phages on the established biofilms, we used scanning electron and confocal microscopy to determine the physical changes in the properties of the colony biofilms. The topology of the untreated days 1, 2, 3, and 4 biofilms have evenly distributed cells (**Figure 2A**). In contrast, the surface of the phage-treated day 1 biofilms had distinct lysed zones (plaques, represented by green arrows) of ∼180–300 μm diameter distributed on the surface but intact bacterial cells were observed around the edges of the lysed zones (orange arrows) (**Figures 2B,C**). Under higher magnifications, it was clear that phage lysis was selective for vegetative cells only, leaving the spores (yellow arrows) and spore mother cells intact (blue arrows) (**Figure 2D**). Examination of the lysed zone of day 2 biofilms (**Figures 2E,F**) revealed elongated cells measuring 15–100 μm in length (indicated using a red arrow) and spores (**Figure 2F**). These elongated cells were only present in the lysed parts of the biofilm. Similar lysed zones were seen in the phage treated biofilms after 3 and 4 days (**Figures 2G,H** respectively) but more spores were seen in day 4 biofilm (**Figure 2H**). At day 5, no plaques were observed but spores, spore mother cells and disintegrated cells (indicated using a purple arrow) were observed in the control and phage-treated biofilms (**Figures 2I,J** respectively).

Phage treatment on the biofilm resulted in a ∼0.5–1 log reduction in vegetative cells for 1–4 days old biofilms, but a negligible effect on the day 5 biofilm cells was observed. Spore formation also progressed daily despite phage treatment (**Figure 2K**).

Analysis of live or dead bacteria in the biofilms using differential staining and confocal microscopy confirmed the lysed zones (green arrows) and the unaffected areas around the zones (orange arrows) (**Figure 3A**). In addition, the elongated cells observed in the lysed zones were dead cells as stained by the red propidium iodide dye (**Figures 3B–D**).

Efficacy of Phage Prophylactic and Remedial Regimens on *G. mellonella* Larva

To further test the ability of the phages for prophylactic or remedial treatment for CDI we determined their efficacy in the *G. mellonella* larvae using the same bacterial strain and phage combination used for the biofilm assays.

Prior to the bacteria infection, we confirmed that the BHI medium used to suspend the phages and bacteria had no significant effect on the larvae (Supplementary Figure S2). The stability of the phages within the larvae was determined by administering the phages via the oral cavity and analyzing phage recovery from the feces and gut of the sacrificed insects using spot test. Phages remained stable and recoverable from the insects for up to 5 days (Supplementary Figure S3). To determine the pattern of CD105LC2 colonization within the insects, a range of doses were tested and we showed that 10^5 CFU caused the death of all insects within 60 h, with first fatal case recorded in the first 24 h post-infection (Supplementary Figure S2). As expected, colonization progressed with time and approximately 10^8 CFU/larva of bacteria load was recovered at the end of the treatment as opposed to $\sim 10^4$ CFU/larva starting bacterial load (Supplementary Figure S4).

To determine phage efficacy in this model, 3 regimens were conducted on the insects. Survival and bacterial load were ascertained and compared to untreated group (**Figure 4A**) and to larvae inoculated with phages only (**Figure 4B**). Where the

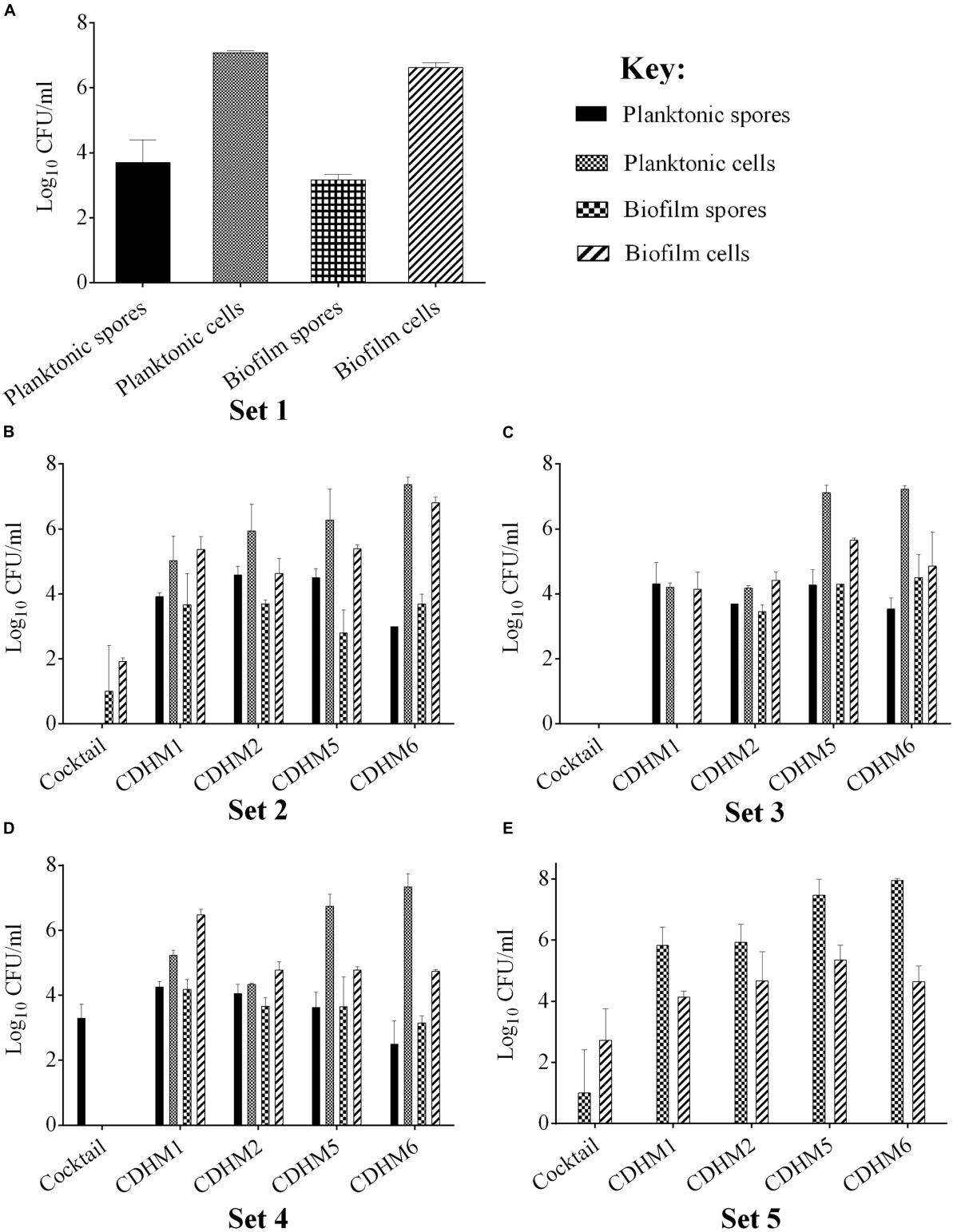

FIGURE 1 | Bacterial recovery from biofilms in pre- and post-biofilm treatments with combined and individual phages (CDHM1, 2, 5 and 6). (A) Set 1 represents the untreated biofilms while Sets 2–4 represent the pre-treatment regimens, where the phages were added after bacterial growth for 1 h (Set 2, **B**), phages were incubated for 1 h before bacteria (Set 3, **C**) or mixed together before adding to plates (Set 4, **D**). **(E)** Represents viability counts after phage treatment post-biofilm formation. Experiments were conducted with three replicates and repeated twice. Data was analyzed using GraphPad Prism 6. Error bars are SEM of all replicates.

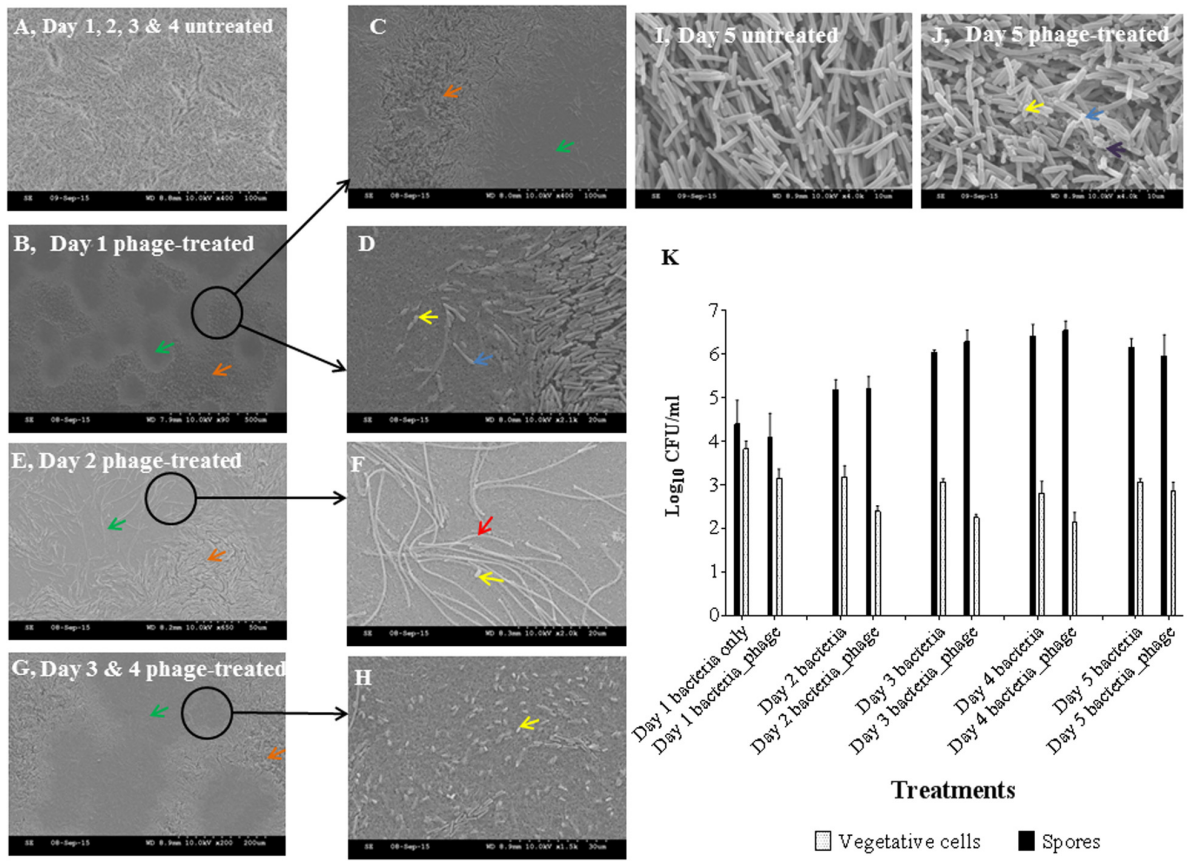

FIGURE 2 | Scanning electron microscopy (SEM) analyses and bacterial enumeration from established *C. difficile* (CD105LC2) colony biofilms treated with phage combination. (A) Topology of untreated biofilm. Impact of phage cocktail on topologies of Day 1 **(B–D)**, Day 2 **(E,F)**, Day 3 **(G)** and 4 **(H)** and Day 5 (untreated **I** and treated, **J**) biofilms are shown. Green arrows, plaques (lysed zones); orange arrows, unaffected areas; blue arrows, spore mother cells; yellow arrows, spores; red arrow, elongated cells, and black arrows, disintegrated cells. Graphical representation of viability counts for spores and vegetative cells are shown in **(K)**.

insects were treated with phages 2 h before inoculating them with the bacteria, the insects were completely protected with 100% survival rate within this group (**Figures 4C** and **5A**). This is comparable to the phage control group (**Figure 4B**). When the larvae were given both the phages and the bacteria simultaneously, efficacy was reduced to 98, 85, and 72% at the 36, 48, and 60 h respectively post inoculation (**Figures 4D** and **5A**). The least effective treatment was recorded in the remedial group where phages were added 2 h post-bacteria. Infection resulted to 82, 65, and 30% survival at the three time points (**Figures 4E** and **5A**). All larvae were dead at the end of the experiment, however, (**Figures 4F** and **5A**). The bacterial numbers at the end of the experiments were consistent with results from the survival assays (**Figures 5B,C**). The prophylactic group (Phages, CD105LC2) had the lowest bacterial load with $\sim 10^2$ CFU/larva recovered while $\sim 10^6$ CFU/larva were observed with the remedial group and $\sim 10^4$ CFU/larva when phages/bacteria were administered simultaneously (**Figure 5B**). The treatments were statistically significantly different ($P < 0.0001$). A similar trend was observed with the qPCR data (**Figure 5C**). Sixty isolates were recovered from all the treatment and testing them with the phage combination showed that the isolates were still sensitive to the phages.

Effect of Multiple Phage Doses on *C. difficile* Colonization and Survival in the *Galleria* Model

In an attempt to improve the efficacy of the phages administered post-infection, we determined if the use of multiple phage doses could improve the remedial treatment. This was indeed the case and larvae given a single dose and 2 doses had the lowest survival rates of ∼10 and 20% respectively, while groups that received 3, 4, or 5 doses showed an increasing improvement with survival rate of ∼60% at the end of the experiment (**Figure 6A**). Treatments differences here were also statistically significant ($P < 0.0001$). Again bacterial recovery from the larva was consistent with decreased bacterial numbers as the number of phage dosages increased. Approximately, 10^5 CFU/larva were recovered from the single dose group as opposed to 10^2 CFU/larva from the group, which received five doses (**Figure 6B**).

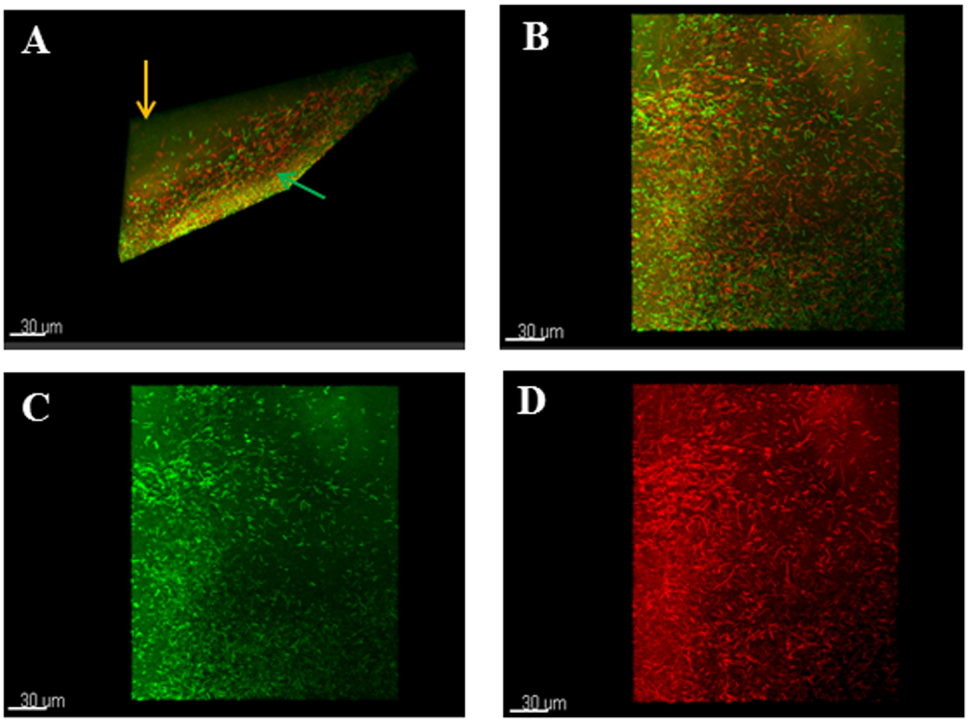

FIGURE 3 | Confocal microscopy analyses of stained phage-treated colony biofilms. (A) T-section through a 24 h colony biofilm treated with phages and stained with Live/Dead viability staining kit showed lysed zones (green arrow) and unaffected areas (orange arrow). **(B)** Shows mixture of live/dead cells in the biofilm. **(C)** Shows normal bacterial morphology of the live cells. **(D)** Shows elongated dead cells in the biofilm. Biofilms were treated with Live/Dead Viability Kit, which stained live cells green and the dead cells red by the Syto 9 and propidium iodide dyes respectively.

Phage Prophylactic Regimens and Phage/Antibiotic Treatments

We showed that the phages remain viable within the *Galleria* heamolymph for up to 5 days. In experiments shown in **Figure 5**, phages were administered 2 h prior to bacterial infection. Here, to determine the impact of giving phages to insects for longer times before bacterial inoculation prophylactic periods of 2 h (A), 6 h (B), 12 h (C), and 24 h (D) prior to bacterial infection were examined. Interestingly, increased phage activity resulted in increased phage counts in the 2–12 h delay times and corresponding decreased bacterial counts as the time delay prior to bacterial infection increased ($\sim 10^2$ CFU/larva at 24 h delay as opposed to $\sim 10^3$ CFU/larva at 2 h delay) (**Figure 7A**).

When the phages were tested as adjuncts to vancomycin, but given prior to bacterial challenge, vancomycin was less effective at removing *C. difficile* (with $\sim 10^2$ CFU/larva cells recovered) (E) than if phages were not given. In contrast, when vancomycin was given prophylactically before infection, the phages were more effective, leading to only ∼10 CFU/larva recovered and this therapy (F) is more effective than vancomycin treatment (G), vancomycin/phage prophylaxis (H) and remedial treatment (I) in which $\sim 10^2$ CFU/larva were recovered in both regimens (**Figure 7A**).

To mimic dysbiosis, clindamycin was given to the insects before bacterial infection followed by phage treatment (J). Here, we saw a proliferation of bacterial growth with $\sim 10^5$ CFU/ml of bacteria being recovered. However, if phages were given post-clindamycin treatment and before bacterial infection (K), only $\sim 10^2$ CFU/larva of bacteria were detected, which is comparable to the combined phage/vancomycin prophylaxis (**Figure 7A**).

In terms of survival, larvae in groups A, B, C, D, E, F, H, I, and K all survived but ∼10 and 30% of the larvae died in the vancomycin treatment and clindamycin pre-treatment 1 (G and J) respectively (**Figure 7B**).

DISCUSSION

Clostridium difficile is a notorious nosocomial pathogen causing fatalities in the immunocompromised. A limiting factor to the control of the disease is the lack of treatment options (Zucca et al., 2013; Hargreaves and Clokie, 2014). *C. difficile* produces spores and biofilms that significantly contribute to its virulence by enabling persistence and proliferation in the environment and gut, and resistance to antimicrobial and cleaning agents (Akerlund et al., 2008; Burns et al., 2011; Burns and Minton, 2011; Dawson et al., 2012; Đapa et al., 2013). Phages are antimicrobial agents that can penetrate biofilms and could potentially be developed to supplement the currently available CDI treatments for better efficacy and clinical outcome (Cos et al., 2010; Morten et al., 2011). Here, we examined the impact of an optimized phage combination on *C. difficile* biofilms and tested their efficacy both as a stand-alone treatment and as adjunct to vancomycin

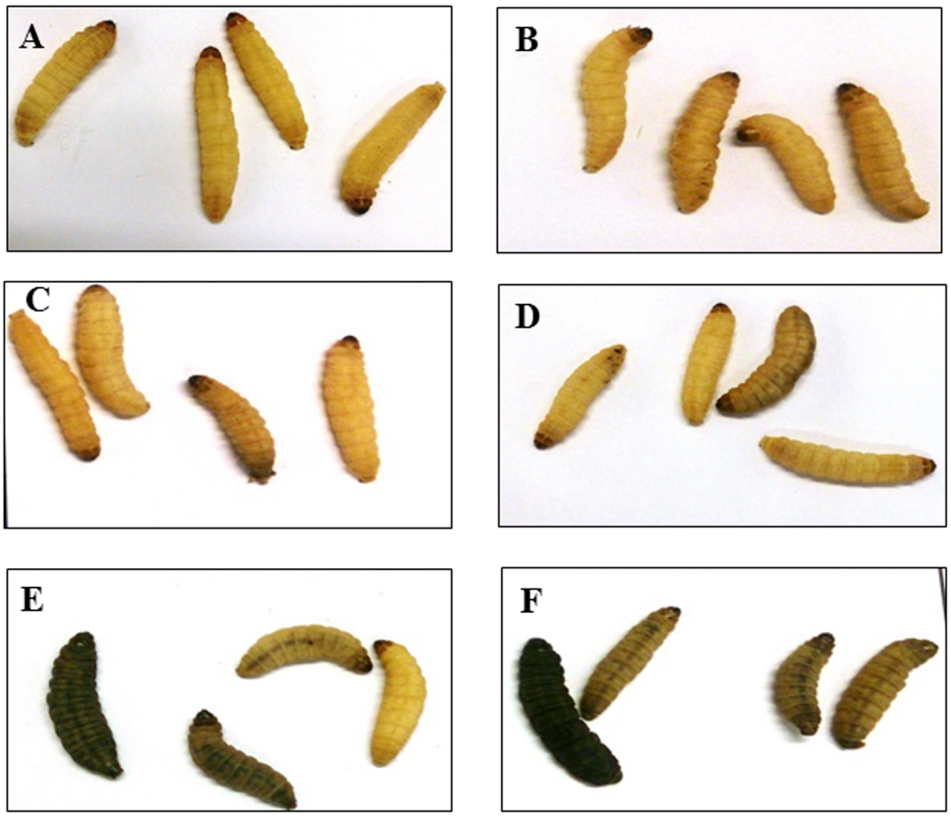

FIGURE 4 | Impact of phage treatment on the morphology and survival of *G. mellonella* larvae at the end of 60 h. Larvae were treated with either 10^5 CFU of bacteria and/or 10^6 PFU of phages. The untreated **(A)**, phage-only inoculated larvae **(B)** and prophylaxis group **(C)** remained healthy and yellow in color at the end of the experiment. While some of the larvae in groups which received a mixture of phages and bacteria **(D)** and the remedial group **(E)** turned black in color. All larvae in the control bacterial group **(F)** turned black in color at the end of the treatment. Larvae are considered dead when they turn black and remain motionless.

in a *G. mellonella* larva CDI model. We showed that the phages can penetrate well-established *C. difficile* biofilms leading to lysis, plaque formation and a reduction in bacterial viability and biomass *in vitro*. A single phage treatment is effective in the pre-biofilm treatment *in vitro*, and prophylactically in the insect models, although multiple phage doses were needed to improve the remedial regimen.

C. difficile Phages Could Target Biofilms of a Prevalent Ribotype 014/020 Strain

To evaluate the potential efficacy of our phages for CDI treatment, we targeted a clinically prevalent and severe ribotype strain (CD105LC2, ribotype 014/020) (Indra et al., 2015; Lessa et al., 2015). In addition to the pathogenicity of CD105LC2, our previous data showed that the individual phages in the mix have optimal plaquing efficiencies on the strain and this combination could completely eradicate *C. difficile in vitro* and reduce its colonization in a CDI hamster model (Nale et al., 2016). Having previously established the efficacy of the phages on *C. difficile in vitro* and on a hamster model, here we went further to test their effect on the biofilm and insect models. This is to determine if they are useful models in which to probe different aspects of *C. difficile*-phage interaction which can inform their future development, and of course useful models for the development of other phages that target biofilm-producing bacteria.

Optimized Phage Combination Is More Effective at Removing *C. difficile* Biofilms Compared to Single Phage Treatments

When testing the efficacy of the phages on biofilms we showed that the phage combination is more efficient at reducing biofilms compared to using the single phages. This observation concurs with previous findings, which showed that treatment of *C. difficile* with single phage is commonly associated with the emergence and proliferation of resistant or lysogenic clones (Ramesh et al., 1999; Meader et al., 2010, 2013; Govind et al., 2011; Nale et al., 2016). Like all previously characterized *C. difficile* phages, the phages examined in this study have integrases in their genomes and could potentially access the lysogenic pathway (Goh et al., 2013; Hargreaves and Clokie, 2014). However, our previous data using the optimized phage combination *in vitro* suggest that the effect of resistance or lysogeny could be circumvented through complementation (Nale et al., 2016). The data presented here further supports this observation as the combined phages caused clear patches of killing on the biofilms.

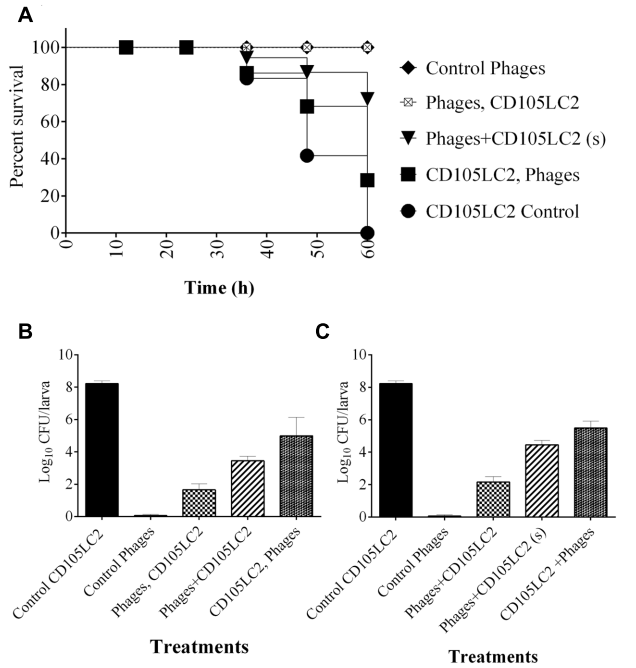

FIGURE 5 | Impact of phage treatment on *C. difficile* colonization and survival rates of *G. mellonella* larvae CDI model. (A) Survival curve showing impact of phage treatment on the larvae in the prophylaxis group (phages, CD105LC2), remedial (CD105LC2, Phages) regimens or larvae treated with a mixture of phages and bacteria simultaneously (phage + CD105LC2 (s)). The treatments were compared with control groups where larvae were given phages only (Control phages) and those that received bacteria only (CD105LC2 control). Bacterial enumeration on CCEY plates are shown in **(B)**, while **(C)** represents qPCR absolute quantification data targeting *C. difficile* 16S rRNA and comparing with standard CD105LC2 curve. A statistical significant difference in time to death was observed ($p < 0.0001$) using four larvae per group and experiment was repeated three times. Error bars are SEM of all the replicates. Data was analyzed using GraphPad Prism 6.

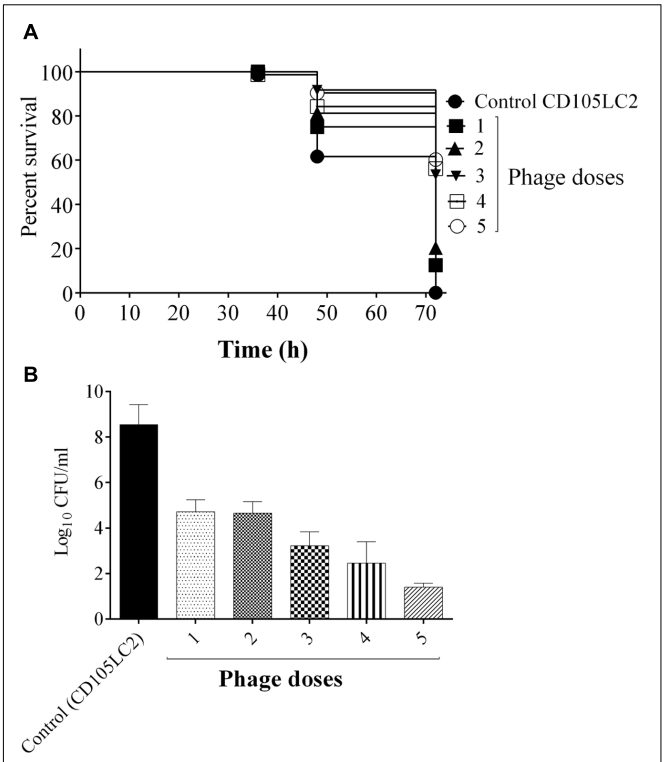

FIGURE 6 | Impact of multiple phage doses on *G. mellonella* larvae CDI model. Larvae were treated with a mixture of bacteria and phages at the beginning of the experiment (one dose). Subsequently, the larvae received single increment phage doses at 2, 6, 12, and 24 h, which represent 2, 3, 4, and 5 doses respectively. Impact of phage treatment on larvae which received all the dose regimens at the time points are presented. **(A)** Survival rates of larva treated with the different doses. **(B)** Bacterial enumeration from the larvae at the end of the experiment. Four larvae were examined in each group and experiment was repeated three times. A statistical significant difference in time to death was observed ($p < 0.0001$). Error bars represent SEM of all replicates. Data was analyzed using GraphPad Prism 6.

C. difficile Phages Are Effective Pre-biofilm Agents

We investigated three different pre-biofilm treatments with the combined phage mixture. In all cases, a significant biofilm reduction was observed. The clearance could be attributed to the ability of the phages to either completely eliminate or reduce the bacteria even before the biofilms were formed (Chan and Abedon, 2015; Nale et al., 2016). When the phages were incubated at 37°C for an hour before the bacterial cultures were added (Set 3), bacteria were eliminated beyond limit of detection. Similar observation was made when catheters were pre-treated with phages before exposure to *Pseudomonas aeruginosa* biofilm (Fu et al., 2010). This could be attributed to the phages being able to acclimatize to the incubation temperature and conditioned to optimum bacteriolytc activity before the bacterial culture was added (Taj et al., 2014). Temperature plays key roles in the attachment, adsorption, latent period, and multiplication of phages, and thus could greatly affect their infectivity (Olson et al., 2004; Jończyk et al., 2011). Efficacy was not as significant in Sets 2 and 4, where the phages were added directly from storage (at 4°C) to the culture at 37°C. Although the phages maintain a stable latent period in storage, at lower than optimal bacterial growth temperature, reduced attachment may take place leading to decreased phage activity (Jończyk et al., 2011). The temperature change encountered by the phages after being transferred to 37°C could have retarded their initial activity, allowing the bacteria to multiply and thus led to reduced MOI, and consequently recovery of bacteria in the phage-treated culture. The reduced effect on the established biofilm could potentially be attributed to less preference of phages to mature biofilms (Abedon, 2016) or the negative impact of PBS used for the biofilm washings on the activity of the phages (Silva et al., 2014). Our preliminary data showed that the stability of the phages in PBS is greatly reduced by ∼ 2 logs CFU/ml by 1 h (Supplementary Figure S5). This observation is in agreement with a previous report (Silva et al., 2014).

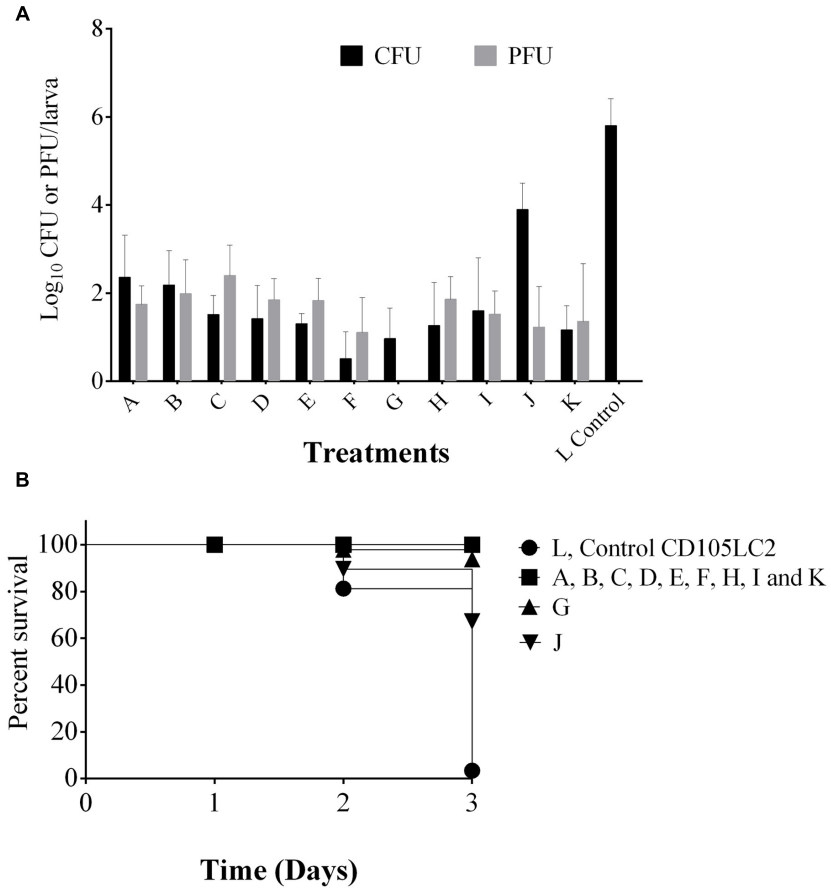

FIGURE 7 | Analysis of phage prophylactic regimens and impact of combined phage/vancomycin treatment on the *G. mellonella* larvae. (A) Shows phage and bacterial recovery after prophylactic treatment with phages and delays of 2, 6, 12, and 24 h in groups A, B, C and D respectively were observed before bacterial exposure. Group E received phage followed by bacteria and vancomycin at the 2nd and 4th hour respectively. Group F was a reverse of group E. Group G, is vancomycin remedial treatment. H group represents combined phage/vancomycin prophylaxis and I group is combined phage/vancomycin remedial regimen. J and K regimen mimic dysbiosis where clindamycin was given first followed by bacteria then phage in J, but phages were given before bacteria in K. Larvae in bacterial control group L received only bacteria. **(B)** Represents survival rates in the treatments. Four larvae were examined in each group. Experiment was repeated three times. Error bars represent SEM of all replicates. Data was analyzed using GraphPad Prism 6.

Phages Could Impact Established *C. difficile* Biofilms

Although we observed that the phages impacted the established biofilms less than the pre-treated cultures, confocal and scanning electron microscopy data both showed that the phages did penetrate the – biofilms and clear phage plaques were formed (**Figure 2**) (Abedon, 2016). In addition to the microscopy observations, further evidence to suggest that the phages penetrate the biofilm was indicated by the reduction in the vegetative cell counts on up to day-4 biofilms. Sometimes, phage lysis of biofilms is mediated by certain phage proteins which could chemically degrade the extracellular polymeric substance (EPS) found in the biofilm (Chan and Abedon, 2015). Previous work conducted on *C. difficile* phages has shown that purified endolysin is effective at clearing of the bacteria. Evidence to suggest its activity on *C. difficile* biofilms is yet to be confirmed (Mayer et al., 2008; Mayer and Narbad, 2012). Data from our SEM analysis revealed that the clearance was restricted to healthy vegetative cells only, thus we could attribute the clearance to lysis from phage infection (Chan and Abedon, 2015; Abedon, 2016).

The observation of elongated dead cells in the lysed zones is consistent with other findings that associated bacterial elongation or plasticity with loss of normal cell division process, changes in metabolic processes or DNA damage (Justice et al., 2008; Ghaffar et al., 2015). This morphological change is associated with stress conditions. Thus, conditions such as antimicrobial treatments or starvation and have been reported in bacteria such as *Helicobacter pylori*, *Campylobacter* and *Listeria monocytogenes* (Takeuchi et al., 2006; van der Veen et al., 2010; Ghaffar et al., 2015) and could trigger cell lysis (Kohanski et al., 2010). In *C. difficile*, cell elongation was reported to be associated with stress from treatment of the cells with sub-MIC of ridinilazole, which caused the cells to increase from 4 μm to 100 μm in length after 24 h treatment with the antibiotic (Bassères et al., 2016). Although the mechanism of ridinilazole is not clear, this response has been attributed to the SOS response and cell death

from activity of β-lactam antibiotics (Kohanski et al., 2010). This bacterial response to antimicrobial stress, leading to cell elongation and death could possibly explain our observation of filamentous cells at 24 h.

Phages Are Effective Prophylactic Therapeutics and Adjuncts to Vancomycin in *G. mellonella* CDI Model

We investigated the efficacy of the combined phages alone and as adjuncts to vancomycin in *G. mellonella* larvae. Although the insect is used as an infection model for various pathogens (Mukherjee et al., 2010; Ramarao et al., 2012; Hill et al., 2014; Beeton et al., 2015) and antimicrobial testing (Seed and Dennis, 2009; Abbasifar et al., 2014; Beeton et al., 2015; Olszak et al., 2015), to our knowledge this report is the first published data to describe its use to study *C. difficile* colonization and phage treatment. We established that 10^5 *C. difficile* cells were sufficient for colonization to occur in the larvae and to cause the first and total mortality at 24 and 60 h respectively post-infection. The choice of the inoculum dose and timing was optimal to allow phage/vancomycin therapies to take place. Also, the bacterial inocula we used were suspended in BHI broth. Previous reports showed that bacteria were suspended either in PBS (Ramarao et al., 2012; Hill et al., 2014) or in 0.9% NaCl (Mukherjee et al., 2010, 2013). However, we could not use either of these solutions since our phages are unstable in PBS, and the NaCl solution negatively interfered with bacterial growth. Therefore, the bacteria were re-suspended in cold BHI broth, which is the growth medium for the bacteria and in which the phages are stable. The BHI broth control is comparable to PBS and NaCl as shown by the larval survival data (Supplementary Figure S2).

We conducted our phage treatment at MOI of 10 to allow comparison of the data from the current study with our previous report (Nale et al., 2016). Work is ongoing to examine other MOIs. Although previous work reported the inoculation of bacteria/phages by intrahaemocoelic injection (Abbasifar et al., 2014; Beeton et al., 2015), we found ingestion via the oral route more efficient at inducing colonization and producing reproducible therapy results. The observed higher efficacy of the phages when used prophylactically rather than with remedial treatment is consistent with a previous report (Beeton et al., 2015). Interestingly, the best treatment was observed when vancomycin was given prophylactically followed by phage application. This is significant as it could potentially serve as an intervention therapy to supplement vancomycin and prevent disease relapse. Similarly, the effectiveness of increased window time of having phages prior to bacterial infection could also be attributed to the ability of the phages to be distributed throughout the gut and acclimatize to the incubation temperature (Jończyk et al., 2011; Beeton et al., 2015) for effective bacteriolysis. The increased survival rates and corresponding decreased bacterial counts observed with the multiple phage dose regimens could be attributed to increased number of phages consequently leading to increased MOI and activity.

CONCLUSION AND RECOMMENDATIONS

The optimized phage combination studied here showed great potential in the control of *C. difficile* biofilms. And the biofilm model is useful for the development of phages that target both this and other pathogenic bacteria. Pre-treating likely surfaces, or potentially, cell lines is most effective as no colonization occurred after their application. Again in the insect model, the phages demonstrated a clear prophylactic potential as a stand-alone treatment for CDI and as adjuncts to vancomycin in the remedial treatment. These characteristics of the phages could be explored further to prevent and control CDI.

AUTHOR CONTRIBUTIONS

JN, MC, and MRJC designed the experiments. JN, PC, and PH performed the biofilm assays. JN, MC, and PH performed the *in vivo* experiments. JN analyzed the data. JN, MC interpreted the results. JN drafted the manuscript. MC, PH, PC, and MRJC edited the manuscript. JN, MC, PC, PH, and MRJC agreed to be accountable for all aspect of the manuscript and approved the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01383

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Characterization and Testing the Efficiency of *Acinetobacter baumannii* Phage *vB-GEC_Ab-M-G7* as an Antibacterial Agent

*Ia Kusradze[1], Natia Karumidze[1], Sophio Rigvava[1], Teona Dvalidze[1,2], Malkhaz Katsitadze[1], Irakli Amiranashvili[3] and Marina Goderdzishvili[1]**

[1] G. Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilisi, Georgia, [2] Ivane Javakhishvili Tbilisi State University, Tbilisi, Georgia, [3] Institute of Medical Research, Ilia State University, Tbilisi, Georgia

Acinetobacter baumannii is a gram-negative, non-motile bacterium that, due to its multidrug resistance, has become a major nosocomial pathogen. The increasing number of multidrug resistant (MDR) strains has renewed interest in phage therapy. The aim of our study was to assess the effectiveness of phage administration in *Acinetobacter baumannii* wound infections in an animal model to demonstrate phage therapy as non-toxic, safe and alternative antibacterial remedy. Using classical methods for the study of bacteriophage properties, we characterized phage *vB-GEC_Ab-M-G7* as a dsDNA myovirus with a 90 kb genome size. Important characteristics of *vB-GEC_Ab-M-G7*include a short latent period and large burst size, wide host range, resistance to chloroform and thermal and pH stability. In a rat wound model, phage application effectively decreased the number of bacteria isolated from the wounds of successfully treated animals. This study highlights the effectiveness of the phage therapy and provides further insight into treating infections caused by MDR strains using phage administration.

Keywords: ***Acinetobacter baumannii,*** **bacteriophage, phage therapy, animal model, wound infection**

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****Correspondence:***
Marina Goderdzishvili
mgoderdzishvili@pha.ge

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INTRODUCTION

Acinetobacter baumannii is a gram-negative, non-motile bacterium that has become a major nosocomial pathogen due to its multidrug resistance. *A. baumanni* strains have been isolated which are resistant to almost all antibiotics, including a high prevalence of resistance to carbapenems which has been reported worldwide since the 1990's (Kempf and Rolain, 2012; Ahmed et al., 2016; Nowak and Paluchowska, 2016). Most of the strains are still sensitive *in vitro* to colistin, an antibiotic which was considered toxic for a long time (Montero et al., 2004). Recent studies suggest it can actually be used as an efficient antimicrobial agent (Michalopoulos and Falagas, 2011). But colistin resistant *A. baumannii* strains have already been reported (Lopez-Rojas et al., 2011; Kempf and Rolain, 2012; Gupta et al., 2016; Yilmaz et al., 2016). Currently there is no remedy to effectively remove *A. baumannii* from the hospital environment, as this microbe is resistant to dehydration, chemical sanitizers and detergents (Yang et al., 2010). As a result, the risk of *A. baumannii* nosocomial infection is increasing.

The worldwide spread of MDR strains of a number of different pathogens has renewed interest in bacteriophage therapy. Bacteriophages are viruses which infect and lyse bacteria. Lytic phages for

the treatment of infections were first introduced by Felix d'Herelle in 1920 (Matsuzaki et al., 2005). Due to their observed efficacy and recognized specificity, phages have been used as a treatment modality in the Formers Soviet Union and Eastern Europe, especially in Georgia, for decades; much successful work was also carried out in France and elsewhere (Matsuzaki et al., 2005; Sulakvelidze, 2005; Kutter et al., 2010).

The study of phage therapy in animal models is an essential bridge between *in vitro* and clinical studies. Mice with artificial burns, pneumonia, pulmonary infections with various microbes (*Pseudomonas* spp., *Staphylococcuss* spp., *Enterococcuss* spp., *Klebsiella pneumoniae, Escherichia coli*) were successfully treated with phages (Biswas et al., 2002; Chibani-Chennoufi et al., 2004; Vinodkumar et al., 2005; McVay et al., 2007; Debarbieux et al., 2010; Morello et al., 2011). Although several *A. baumannii* phages have been isolated and characterized in terms of potential therapeutic application (Soothill, 1992; Yang et al., 2010; Popova et al., 2012), a very few *in vivo* trials for *A. baumannii* phages have been reported: Wang et al. (2016), showed successfully use of phage intranasal application for treatment pneumonia on mice model. All mice survived after intranasal application of phages published by Jeon et al. (2016) as well. Uncontrolled diabetic rats were used for infected wound modeling for study phage therapy effectiveness by Shivaswamy et al. (2015), where ones more was demonstrated *A. baumannii* phage prospects for treatment of MDR bacteria caused infections. Several studies done in Georgia at Eliava Institute of BMV highlight phage therapy advantages and effectiveness, as well (Kutateladze and Adamia, 2008, 2010). Furthermore, a range of phages targeting the organism in question are required to successfully develop a phage therapy approach. In our study we have characterized a promising new lytic *A. baumannii* bacteriophage *vB_Ab-M-G7* and report its potential in phage therapy on a rat wound model, using both its original and a French clinical strain of *A. baumannii*.

MATERIALS AND METHODS

Bacterial Strains, Identification

Clinical isolates of *Acinetobacter baumannii G7* and *T-10* were used in this study. *A. baumannii* strains *G7* –isolated in Georgia from an injured soldier during the Georgian–Russian War in 2008 as previously described (Kusradze et al., 2011), was used for isolation and concentration of the reported phage. Strain *T-10* was isolated from a patient in the hospital de la Timone, Marseille, France. Bacteria were grown at 37°C in Brain Heart Infusion broth and agar, and in Herellea Agar (Jawad et al., 1994). Matrix-assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Autoflex, Bruker Daltonics) with the flex control software (Bruker Daltonics) was used for identification these strains. A score value >1.9 is considered adequate for identification at the species level (Seng et al., 2009).

Phage Isolation, Concentration, Purification

Bacteriophage was isolated from sewage water. After overnight incubation of the sewage samples with microbial culture in Brain Heart Infusion Broth (BHIB) at 37°C, samples were centrifuged at 5000 *g* for 20 min and filtrated and the supernatant was checked for phages by a standard spot test: overnight host strain (18–24 h.) diluted in BHIB 10^8 cfu ml^{-1} were streaked onto a Petri plate with 1.5% agar. After drying of the lines (15–20 min), 0.05 ml of each supernatant was spotted onto each line. Plates were incubated at 37°C for 18 h. After incubation, the appearance of lysis zones on the bacterial lines shows the presence of phages (Garbe et al., 2010; Karumidze et al., 2013). Phage concentration and plaque morphology was determined by serial dilution of the phage lysate, 1 ml serial diluted phage and 0, 1 ml indicator bacteria was added to 4.5 ml 46°C 0.7% soft-agar and poured onto the 1, 5% bottom agar on Petri dishes. After 30 min the plate was incubated at 37°C. The results were counted after 18–24 h. Plaque Purification of bacteriophage was repeated 15–20 times. Purified phage was amplified and titer in the filtrate was assessed by using the double-agar layer method (Lin et al., 2010). The phage lysate was stored at 4°C.

Phage Host Range Spectrum, Single Step Growth Curve, Adsorption Rate

The phage host range spectrum was determined on 200 *A. baumannii* strains (Eliava Collection) using a standard spot test protocol (Garbe et al., 2010; Karumidze et al., 2013). To determine phage growth characteristics (latent period, burst size), experiments were carried out according to the previously described (Drulis-Kawa et al., 2011; Rigvava et al., 2013), with some modifications. *A. baumannii* strain was grown in 10 ml BHIB until the exponential growth phase, 0, 1 ml phage with titer 10^8 pfu ml^{-1} was added at a multiplicity of infection (MOI) of 0.1. Samples were taken periodically for the determination of phage growth parameters.

To calculate the phage adsorption rate, 1 ml bacterial suspension (10^7 cfu ml^{-1}) and 1 ml phage lysate (10^8 pfu ml^{-1}) were mixed and incubated at 37°C. 0.1 ml samples were taken at 0', 5', 7', 10', 15', and 20' min and added to 9,9 ml BHIB and 0,4 ml chloroform. Samples were mixed well and plated using the double agar-layer method to determine the amount of unabsorbed phages.

pH, Chloroform and Thermal Stability Tests

The phage preparation (1×10^{10} pfu ml^{-1}) was incubated at 37, 50, and 70°C for 24 h. From chloroform stability tests, 1 ml (1×10^{10} pfu ml^{-1}) bacteriophage was mixed with 0.4 ml chloroform and incubated for 24 h at room temperature. For pH stability studies, phage at 1×10^{10} pfu ml^{-1} was incubated at pH 3, 5, 7, 9, and 11 for 24 h. For all three experiments samples were taken at 5 and 24 h and the phage titer was determined using the double-agar-layer method. BHI broth and BHI agar were used.

Electron Microscopy

Phage morphology was determined by transmission electron microscopy as following: a 5 μl sample was placed onto a fresh glow discharged pioloform coated grid; the excess sample was removed and the grid was washed using 2 × DDW (Double Distilled Water); 2 drops of 1% uranyl acetate were added, the excess was immediately removed and the grid was allowed to air dry. Samples were viewed on a JEOL JEM 1400 TEM with an accelerating voltage of 80 kV. Images were captured using a Megaview III digital camera with iTEM software.

Phage DNA Isolation, Restriction Endonuclease Analysis, PFGE

Before phage DNA isolation, the phage lysate was treated with DNAse and RNAse to remove residual bacterial debris. Standard PCR (Polymerase chain reaction) was used to verify the purity of the phage lysate with universal primers (536F CAGCAGCCGCGGTAATAC, Rp2 ACGGCTACCTTGTTACGACTT) targeting the 16S rRNA gene as previously described (Bittar et al., 2008). Phage DNA isolation was performed by using High Pure Viral Nucleic Acid Qiagen Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. For digestion of phage DNA 8 different restriction endonucleases (BamHI, EcoR I, EcoRV, HindIII, HincII, PstI, DpnI, SpeI) were used according to the instructions provided by manufacturer. For separation of the DNA fragments, electrophoresis was done using 0.8% agarose gel. Restriction digestions were repeated three times. Pulsed-field gel electrophoresis was performed as described in Lin et al. (2010) for determination of the phage DNA size.

Preparation for Animal Wound Model

Animal experiments were done according to the Animal Rights Committee in Georgia, which fully recognizes the Universal Declaration of Animal Rights.

A total of 30 adult rats weighing 200–300 g were used in this study. Experimental wounds were done as following: each rat was anesthetized and secured to the operating table. After being shaved, the skin was cleaned in aseptic conditions with a 5% iodine solution and a dorsal full-thickness 1.5 × 1.5cm surgical wound was administered. Interrupted stitches were used to secure a plastic cover to avoid contamination, as well as for procedures which were performed on the animals daily.

Acinetobacter baumannii T-10 and *G7* were used for infection and phage *vB-GEC_Ab-M-G7*was applied as a therapeutic remedy. *A. baumannii G7* was the original host for phage *vB-GEC_Ab-M-G7*, titer on this strain was 1×10^{10} pfu ml^{-1}. *A. baumannii T-10* was chosen randomly from the strains sensitive to phage *vB-GEC_Ab-M-G7*, phage titer on this strain was 2×10^8 pfu ml^{-1}. Phage was diluted to receive 5×10^7 pfu ml^{-1}.

Experimental Animal Wound Model

The 30 rats with experimental wounds were divided randomly into six groups, each containing five rats. Group I was aseptic wound modeling – no bacteria were added, no phage. Group II tested phage therapy of the aseptic wound (therapeutic dose) with 1ml phage application (5×10^7 pfu ml^{-1}), no bacteria were added. Group III – infected wound modeling, 1ml of *Acinetobacter baumannii T-10* 5×10^8 cfu ml^{-1} was applied, no phage were added. Group IV tested phage therapy of the wound infected with 1 ml of *A. baumannii T-10* (5×10^8 cfu ml^{-1}), 1ml of phage (5×10^7 pfu ml^{-1}) were applied 12 h after infection. Group V – infected wound modeling, infected with 1 ml of *Acinetobacter baumannii G7* (5×10^8 cfu ml^{-1}), no phage were added. Group VI -phage therapy of the infected wound (1 ml of *A. baumannii G7* 5×10^8 cfu ml^{-1}) with 1ml phage (5×10^7 pfu ml^{-1}) applied after 12 h from infection. Additional, phage *vB-GEC_Ab-M-G7* (for groups II, IV, and VI) were added on the wounds every 24 h for 6 days. Samples were taken before phage application at time points 0' (12'), 24', 48', 72', and 144' hours using a sterile swab. 4.5 ml of BHI broth was added to the swab tube and was first titered for bacterial count and then was filtered (0.22 μm) and titered for phage, according to previously stated methods. The bacterial titer was determined using Herellea agar, to avoid contamination. At the end of the experiment, every animal was euthanized in accordance with the law of animal rights.

RESULTS

Bacterial Strains

Microbial strains *T-10* and *G7* were identified as *A. baumannii* using MALDI-TOF MS, with score of 2.224 and 2.272 respectively.

Phage Properties

Phage *vB-GEC_Ab-M-G7*was isolated from sewage using *A. baumannii G7* as the host. Using phage purification methods, concentration and titration, a pure, high titer (10^{10} pfu ml^{-1}) stock of *A. baumannii* phage was obtained, which had small plaque morphology on Petri dishes using the double-agar-layer method. The study of phage morphology by transmission electron microscopy showed that the phage has an icosahedral head, about 100 nm in diameter and a 120 nm long contractile tail, thus belongs to Myoviridae (Matsuzaki et al., 2005) (**Figure 1**). It was named *vB-GEC_Ab-M-G7* (phage *phi G7*) according to the scheme for the nomenclature of viruses of bacteria (Kropinski et al., 2009).

Host range spectrum studied on 200 *A. baumannii* strains showed that phage *vB-GEC_Ab-M-G7*was able to infect 68% of the *A. baumannii* strains. The latent period of phage *phi G7* was 20 min and the burst size was 120 pfu per infected cell. Most phages (91.1%) were adsorbed within 7 min (**Figure 2**).

Thermal stability experiments showed that phage retained 100% activity after incubation at 37°C and almost 90% of phages were viable after a 24 h incubation at 50°C (**Figure 3**). However, after 24 h at 70°C no active phages were found. Phage *phi G7* was stable after 24 h of chloroform treatment and over a pH range of 3–11 for 5 h; by 24 h the phage titer was reduced to 10^3 pfu ml^{-1} at pH 3, but it remained unaffected within the pH range 5–11(**Figure 3**).

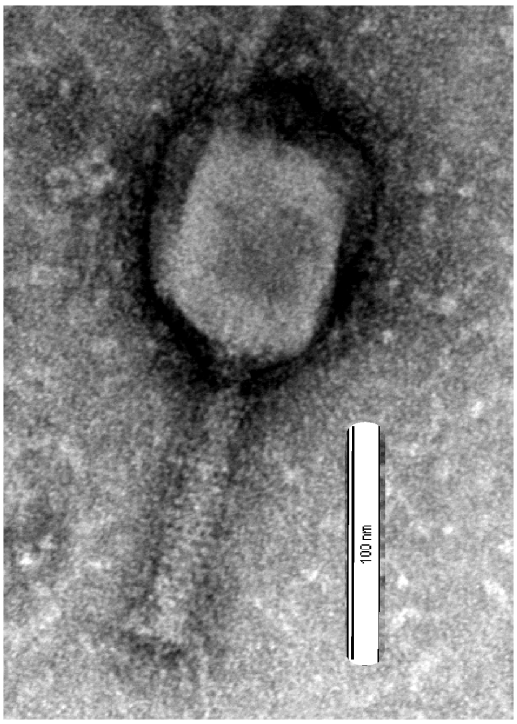

FIGURE 1 | Electron micrographs of phage *vB_Ab-M-G7.* Bar corresponds to 100 nm.

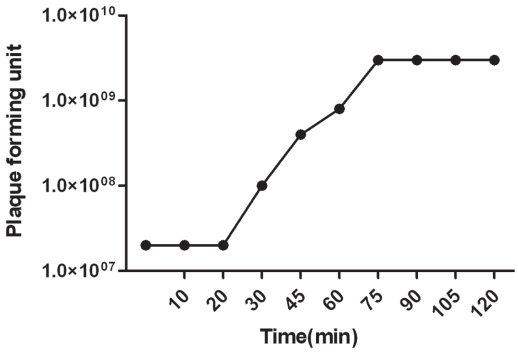

FIGURE 2 | Single step growth curve of phage *vB_Ab-M-G7.* The latent phase takes average 20 min and the phage produces about 120 pfu ml^{-1} per infected cell.

PFGE showed that the phage DNA size was 90 kb. None of the 8 (BamHI, EcoR I, EcoRV, HindIII, HincII, PstI, DpnI, SpeI) restriction endonucleases used in this study digested phage vB_Ab-M-G7, although they did digest the DNA of other phages.

Phage Therapy in Infected Animal Wound Model

All of the Group I animals survived and no *A. baumannii* strains were isolated (grown in Herellae agar) from the samples taken from them, indicating that the wound modeling was done in sterile conditions and this artificial wound did not affect the rats' mortality.

To examine the toxicity of phage, the wounded but not infected experimental Group II was used, where only phage *vB-GEC_Ab-M-G7*was applied to the artificial wound. All of the wounded rats survived, indicating that phage *phi G7* was not toxic. Samples taken from the animals showed no contamination with *A. baumannii* and the titer of the phage was not more than $2 \times 10^1 \pm 1.7$(SD) pfu ml^{-1} in each samples taken during the experiments (**Table 1**).

In groups III and V, the animals were administered 1 ml of *A. baumannii T-10* or *G7* (5×10^8 cfu ml^{-1}). Purulent and inflammatory processes after the second day of infection could be easily observed and became heavier at the end of experiment, indicating of infection and not colonization. Wounds infected with *A. baumannii G7* were observed to be more serious and complicated, and in group V the rate of mortality was 30%, by the fifth day of the experiment. Microbial growth is shown in **Figure 4** and **Table 1**. Samples were tested for phage contamination; no phage plaques were detected in the plated samples from animal groups III and V.

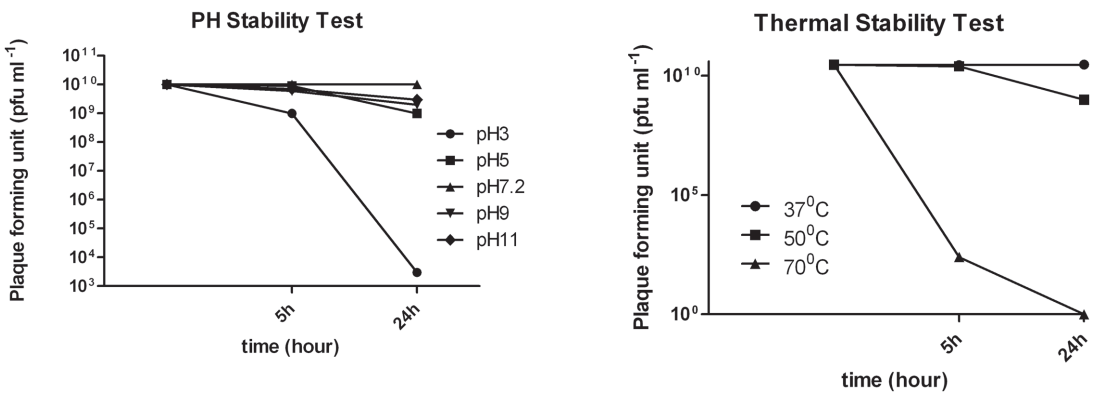

FIGURE 3 | pH and thermal stability of phage *vB_Ab-M-G7*.

TABLE 1 | Alternation of the bacterial titer in phage treated infected wounds.

Groups	Rats (200–300 g)	Bacterial Infection (1 ml 5 × 10^8 CFU" ml^{-1})	Delay Before Phage Application	Phage Application 1 ml 5 × 10^7 PFU ml^{-1} (6 days)	Results
I	Aseptic wound	–	–	–	No strain was isolated
II	Aseptic wound	–	12 h	*Phi G7*	No strain was isolated; 2 × 10^1 ± 1.7(SD)' PFU"' ml^{-1}
III	Infected wound	*A. baumannii T10*	–	–	8 × 10^6 ± 0.9(SD) CFU ml^{-1} increased to 9 × 10^7 ± 1.6(SD) CFU ml^{-1}
IV	Infected wound	*A. baumannii T10*	12 h	*Phi G7*	7 × 10^6 ± 0.9(SD) CFU ml^{-1} decreased to 9 × 10^2 ± 1.3(SD) CFU ml^{-1}
V	Infected wound	*A. baumannii G7*	–	–	1 × 10^7 ± 1.8(SD) CFU ml^{-1} increased to 2,5 × 10^8 ± 2.9(SD) CFU ml^{-1}
VI	Infected wound	*A. baumannii G7*	12 h	*Phi G7*	1,4x10^7 ± 0.9(SD) CFU ml^{-1} decreased to 3 × 10^2 ± 1.9(SD) CFU ml^{-1}

', Standard Deviation; ", Colony Forming Unit; "', Plaque Forming Unit.

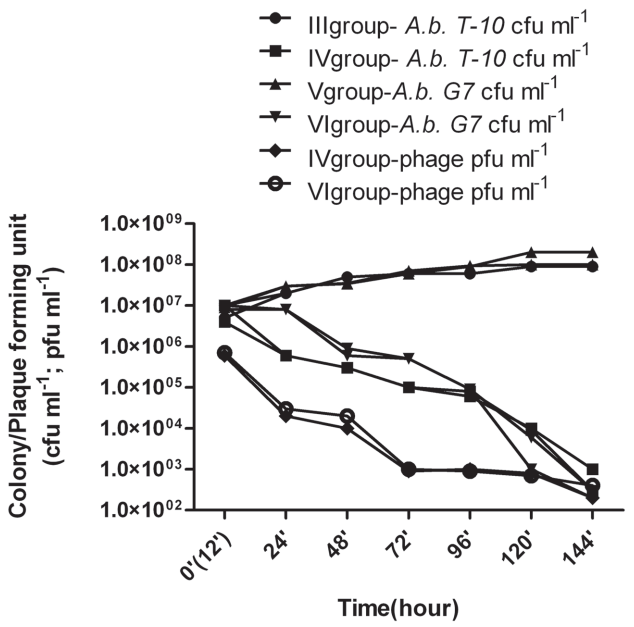

FIGURE 4 | Phage therapy results in animal wound model. *III group* – *A. baumannii T-10* strain titer was increased from ≈8 × 10^6 ± 0.9 (standard deviation – SD) cfu ml^{-1} to ≈ 9 – 10^7 ± 1.6(SD) cfu ml^{-1}; *IV group*- alternation of the bacterial and phage titer during the 7 days were as following, ≈7 × 10^6 ± 0.9(SD)cfu ml^{-1} to ≈ 9 × 10^2 ± 1.3(SD) cfu ml^{-1}; ≈6 × 10^5 ± 1.5(SD) pfu ml^{-1} to ≈ 3 × 10^2 ± 1.6(SD) pfu ml^{-1}, respectively; *V group* – *A. baumannii G7* strain titer was increased from ≈1 × 10^7 ± 1.8(SD) cfu ml^{-1} to ≈ 2.5 × 10^8 ± 2.9(SD) cfu ml^{-1}; *VI group*- alternation of the bacterial and phage titer during the 7 days were as following, ≈1.4 × 107 ± 0.9(SD) cfu ml^{-1} to ≈3 × 10^2 ± 1.9(SD) cfu ml^{-1}; ≈7 × 10^5 ± 1.5(SD) pfu ml^{-1} to ≈2 × 10^2 ± 1.9(SD) pfu ml^{-1}, respectively. Phage and bacteria titer is given in arithmetical average.

Group VI – 12 h after infection with 1ml of *A. baumannii G7* (5 × 10^8 cfu ml^{-1}), 1 ml of 5 × 10^7 pfu ml^{-1} phage *phi G7* was applied to the wound during 6 days. During treatment the rats were active and some of them were aggressive. Before the phage application no inflammatory processes were present. By the third, day purulent processes appeared which fully vanished by the end of the experiment. The reduction of the infection symptoms was correlated with decreasing of the bacteria titer to an average 3 × 10^2 ± 1.9(SD) cfu $ml^{-1}$and the phage titer dropped from 7 × 10^5 ± 1.5(SD) 2 × 10^2 ± 1.9(SD) pfu ml^{-1} (**Figure 4**, **Table 1**). All the animals survived.

Group IV -*A. baumannii T-10* showed less virulence than *A. baumannii G7,* (groups III and V). Slight inflammations, as well as a little purulence were characteristics of the treatment processes, but it fully disappeared before days 5 and 6. Decreasing of the bacterial and phage titer, respectively, are given in **Figure 4** and **Table 1**.

DISCUSSION

Due to the increase in the number of antibiotic resistant microbes, phage therapy is considered as alternative treatment for MDR bacterial infections (Chibani-Chennoufi et al., 2004; Sulakvelidze, 2005; Debarbieux et al., 2010; Kutter et al., 2010). Phage preparations are widely used to treat infections caused by *E. coli, Pseudomonas aeruginosa, Salmonella* spp., *Enterococcus faecium, Streptococcus* spp., *Staphylococcus aureus* and *Proteus* spp. in countries of the former Soviet Union (Matsuzaki et al., 2005; Sulakvelidze, 2005), but *A. baumannii* phages have not yet been used as therapeutic tools. In our study we have shown effectiveness of the phage *vB-GEC_Ab-M-G7in vivo,* correlated with its high *in vitro* activity.

Phage *phi G7* is a tailed virus with 90kb double stranded DNA genomes, belonging to the *Myoviridae* family. Some characteristics of phage *phi G7* are: a short latent period and large burst size, quite wide host range (68% on 200 clinical strains), resistant to chloroform and stabile in different thermal and pH ranges. Resistance to different restriction enzymes presumably helps make this phage active over such a wide

spectrum; sequencing of the phage will determine whether this involves selection against all such restriction sites, as was observed for staph phage SB-1 (Kvachadze et al., 2011) or some other mechanism, such as the substitution of an unusual base seen in coliphage T4 and its relatives (Miller et al., 2003). All these characteristics help made phage *phi G7* a very promising component of a cocktail for treatment of *A. baumannii* infection. For this *in vivo* study we selected two *A. baumannii* strains: *G7* and *T-10* that were correctly identified as *A. baumannii* using MALDI-TOF MS. Patient-delivered *A. baumannii G7* is the host for phage *phi G7* and strain *T-10* was chosen randomly from *phi G7* sensitive strains for this experiment. No phage *phi G7* adaptation procedures were carried out using the *A. baumannii T-10* strain. We wanted to show the effectiveness of the phage *phi G7* therapy in an artificial infected rats wound model and at the same time to investigate potential differences in the therapeutic effectiveness of this phage, for treatment of infections caused by both host and randomly selected *A. baumannii* strains.

We have demonstrated in this animal wound model that the phage application did not have a toxic effect on wounded rats: in phage control group (group II) where phages were administered to the wound all the rats survived, they were feeling calm and no aggression was observed. Phage application in wound infections caused by *A. baumannii T-10* and *G7* effectively reduced the number of bacteria isolated from treated animals and all visible infection symptoms (red, swollen-purulent wound) disappeared (**Figure 4**, **Table 1**). Aggressive behavior of the infected rats fully vanished which was correlated with disappearing of infection symptoms. Rats with infections caused by the original host strain and by a randomly selected strain were treated with the same successful results. Obviously more detailed studies examining the effect of the phage dosage, timing of phage administration, pharmacokinetics will need to be undertaken before *A. baumannii* phages can be used in therapy. However, our characterization of phage *vB-GEC_Ab-M-G7*and animal experimentation illustrates its big potential for treatment of infections. We hope this study will provide further insight on treating infections caused by MDR strains using phage administration.

AUTHOR CONTRIBUTIONS

IK planned given research and analysed obtained results. NK, SR, and TD were involved in phage research. IA and MK performed animal experiment. MG was consulting.

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GENERAL COMMENTARY
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Commentary: Phage Therapy of Staphylococcal Chronic Osteomyelitis in Experimental Animal Model

Stephen T. Abedon *

Department of Microbiology, The Ohio State University, Mansfield, Ohio, USA

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A commentary on

Phage Therapy of Staphylococcal Chronic Osteomyelitis in Experimental Animal Model
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Joshua D. Nosanchuk, Albert Einstein College of Medicine, USA

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Philippe Georgel, University of Strasbourg, France
Victor Krylov, I. I. Mechikov Institute for Vaccines and Sera, Russia

***Correspondence:**
Stephen T. Abedon
abedon.1@osu.edu

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Phage therapy—the use of bacterial viruses as equivalents of antibacterial "drugs"—has been practiced for nearly 100 years. Much, particularly since the 1940s, has taken place in the former Soviet Union. Nevertheless, interest in phage therapy in Western countries has been steadily building since the 1980s. Much of this interest has been in response to growing concerns over antibiotic resistance, though there are concerns, as well, over antibiotic side effects (Langdon et al., 2016). Phage therapy in many cases has been used as a means of treating chronic bacterial infections which may be *tolerant* to antibiotic treatment rather than necessarily also genetically resistant. This antibiotic tolerance is thought to occur predominantly as a consequence of bacterial growth within biofilms (Olsen, 2015). In practice, however, what is going on is that phage therapy has been used to treat bacterial infections against which antibiotics, often following months or years of treatment, have not been successful (e.g., Rhoads et al., 2009; Wright et al., 2009; Miedzybrodzki et al., 2012).

Despite this clinical aspect of phage therapy, pre-clinical studies have not tended to focus experimentally on chronic bacterial infections. There have been exceptions, however, particularly in which a fair amount of time has elapsed between bacterial challenge and start of phage treatment (**Table 1**). Here I focus on the recent study of Kishor et al. (2016), which employs the longest delay of which I am aware, in an animal model, between bacterial challenge and phage application.

Kishor et al. (2016) present a rabbit model of chronic osteomyelitis caused by a methicillin-resistant isolate of *Staphylococcus aureus*. Two rabbits were used to test phage cocktail safety (intraperitoneal delivery of $\sim 10^{11}$ plaque-forming units), four served as untreated controls, 12 were treated with phages after ~3 weeks, and another four were treated with phages after 6 weeks. Cocktails consisted of seven phages and a total of four doses were applied, 2 days between each. Wounds were prepared thusly (p. 89): "…a 2 cm long incision was made at the lateral aspect of distal end of femur and metaphysial area was exposed. With the help of a hand drill, a 5 mm diameter unicortical defect was created…" Following bacterial inoculation, "The incised area was covered by sterile bandages with one stitch at middle of open area." Phage treatment by injection into the infected area, with either 3- and 6-week delays, resulted in negative cultures in 15 cases. The 16th was sacrificed for study prior to completion of phage administration.

I have a few minor criticisms of the study, which the authors note is "preliminary." First, it is difficult to tell just how many bacteria were applied. Second, all control rabbits were sacrificed during week 6, meaning that there were no no-treatment controls for the 6-week-delay experiments. Lastly is the notion of "lysis of bacteria from outside," which I interpret as reference

TABLE 1 | Animal models of human phage therapy with treatment delays exceeding 24 h.[a]

Organism(s) treated	Delay before treatment[b]	Challenge	Consequence	Treatment	Results[c]	References
Escherichia coli	168 h	Force-fed axenic mice with 5×10^7 CFU	Intestinal colonization	10^5 PFU/ml in drinking water	$\sim10^8$ CFU/ml reduced to $\sim10^4$ CFU/ml in feces	Chibani-Chennoufi et al., 2004
Escherichia coli O157:H7	48 h	Force-fed mice with 10^9 CFU	Intestinal colonization	Force fed 10^8 PFU, 1 dose, or 10^{10} PFU, 1 dose or "daily"	$<10^3$ vs. $<10^2$ CFU/g in feces for control (etc.) vs. "daily" treatment, around day 8	Tanji et al., 2005
Mycobacterium avium	168 h	IV 3×10^7 CFU to mice	Continuing replication	IV 8×10^9 PFU or 4×10^7 phage-infected *M. smegmatis* in 100 μl, 1 or 2 doses	~0.5-log fewer CFU in spleen for phage-infected *M. smegmatis*, day 14, no reduction for phage only	Danelishvili et al., 2006
Staphylococcus aureus	96 h	Subcutaneous 10^8 or 10^9 CFU/mouse	Abscess development	Subcutaneous 10^9 PFU[d] in 200 μl, 1 or 4 doses	$\sim10^8$ vs. $\sim10^6$ or $\sim10^4$ CFU per abscess for control vs. treatments	Capparelli et al., 2007
Staphylococcus aureus	240 h	IV 5×10^6 CFU to mice	Non-lethal systemic infection	10^9 PFU in 200 μl[e]	$\sim10^4$ CFU vs. 0 in various organs, day 20	Capparelli et al., 2007
Pseudomonas aeruginosa	96, 240 h[f]	10^8 CFU/ml in mouse drinking water[g]	Gut-derived septicemia	10^{10} PFU in 100 μl orally administered	0, 10, and 66.7% 20-day survival for no, 240, and 96-h treatments	Watanabe et al., 2007
Salmonella enterica	48, 336 h	IV 10^6 or 10^5 CFU, respectively, to mice	Sublethal systemic infection	IV 10^7 PFU in 100 μl	$\sim10^4$ CFU reduced to 0 in various organs	Capparelli et al., 2010
Acinetobacter baumannii, *Pseudomonas aeruginosa*, *Staphylococcus aureus*	96 h	Mouse wounds injected with 100 μl of organisms of one type	Infected wound (diabetes mellitus model)	10^8–10^9 PFU presumably topical following one or more debridements, >10 doses	$\sim10^5$ vs. $\sim10^2$ CFU/swab, day 8, control vs. treatment (*S. aureus*)[h]	Mendes et al., 2013
Mycobacterium ulcerans	792 h	Subcutaneous 3×10^5 CFU into mouse footpad	Footpad swelling with continuing replication	Subcutaneous 10^8 PFU into mouse footpad	$\sim3 \times 10^5$ vs. $\sim3 \times 10^3$ CFU/footpad, day 68, control vs. treatment	Trigo et al., 2013
Staphylococcus aureus (MRSA)	96 h	Intranasal 10^6 CFU/mouse	Nare potentially transient colonization	Intranasal 50 μl of 10^7 PFU/ml, 2 doses	$\sim10^{5.5}$ vs. $\sim10^1$ CFU/g, day 11, control vs. treatment	Chhibber et al., 2014
Staphylococcus aureus	168 h	Sinus inoculation of sheep	Sinusitis model	Sinus inoculation of 100 ml of 2×10^8 PFU/ml, 5 doses	~80% reduction in biofilm biomass, control vs. treatment	Drilling et al., 2014
Acinetobacter baumannii (multi-drug resistant)	48 h	10^8 CFU inoculated into wound of diabetic rats	Abscess development	400 μl of 3×10^9/ml PFU sprayed onto debrided wound	$\sim10^9$ vs. 0 CFU, day 8, control vs. treatment	Shivaswamy et al., 2015
Klebsiella pneumoniae	48, 72 h	Intranasal 10^4 CFU/mouse	Lobar pneumonia	IP, liposome-entrapped phages[i]	$\sim10^5$ vs. $\sim10^3$ vs. 0 CFU, day 5, control vs. 72-h delay vs. 48-h delay[j]	Singla et al., 2015
Staphylococcus aureus (MRSA)	504, 1008 h[k]	Intramedullary injection of rabbits with uncertain number ($\leq5 \times 10^6$) CFU	Chronic osteomyelitis	Intralesional injection of 5×10^{11} PFU, 4 doses	Cure of infection versus (for 504 h delay) lack of cure w/o treatment	Kishor et al., 2016

[a] *CFU, Colony-Forming Units; IP, Intraperitoneal; IV, Intravenous; MRSA, Methicillin-Resistant or Multi-drug Resistant Staphylococcus aureus; PFU, Plaque-Forming Units.*

[b] *Time between initial exposure to bacteria and initial exposure to phages. Shown per study are only those delays of longer than 24 h.*

[c] *Day indicated is post initial bacterial challenge.*

[d] *It is difficult to tell whether the phage administration was or was not made directly to the abscess.*

[e] *Phages were presumably delivered intravenously, though this is not explicitly stated.*

[f] *Phage additions were either 48- to 96-h prior to (96-h treatment) or 48- to 96-h post (240-h treatment) the cyclophosphamide-mediated induction of septicemia.*

[g] *This was followed days later by cyclophosphamide IP injection to induce septicemia.*

[h] *P. aeruginosa saw similar drops without phage treatment though sooner with phage treatment; A. baumanni also saw substantial drops with phage treatment when assaying for colony counts using selective media; mostly analogous though not identical and also more variable results were seen for S. aureus and P. aeruginosa with an otherwise equivalent pig model.*

[i] *Both free and liposome-entrapped phages were administered in this study representing concentrations of 10^9 and 10^7 PFU/ml and multiplicities of infection of 1 and 0.01, respectively.*

[j] *No difference in results was observed between control and 48-h delay in phage treatment w/o liposome entrapment. Note that 5-days post bacterial challenge is 3 and 2 days post 48 and 72-h phage treatment, respectively. The 72-h treatment also was reduced to zero CFU 7 days post bacterial challenge while the no-treatment control was reduced to 10^3 CFU/ml at 7 days.*

[k] *3 and 6 weeks, respectively, though the 3-week treatment may in fact have actually begun on day 16.*

to "lysis from without" (Abedon, 2011) but which the authors appear to be equating, incorrectly, with the concept of inundation (Payne and Jansen, 2001). Inundation therapy generally is assumed to involve bacteria killing though not necessarily also phage replication nor even bacterial lysis.

A much more substantive concern is that these authors have, in essence, made their reported treatment look *too easy*. This is not a criticism of the results presented but instead stems from a much more general question of just what constitutes a chronic bacterial infection as typically treated clinically using phage therapy. Thus, does this rabbit osteomyelitis model, impressive as it appears, come close to actually modeling antibiotic-tolerant chronic bacterial infections?

The authors indicate that chronic osteomyelitis is defined by bone inflammation that has lasted for at least 6 weeks. By contrast, by the time phage treatment has begun in the clinic, chronic infections typically have persisted, often despite ongoing antibiotic treatment, for up to many years, e.g., a median of 43 months as reported by Miedzybrodzki et al. (2012). Differences therefore may exist between chronic osteomyelitis at 6 weeks and that which phage therapists may encounter in the clinic, and particularly so given that there have been delays in treatment until after more conventional approaches have failed.

Drawing on the Kishor et al. (2016) study as well as others presented in **Table 1**, I would like to suggest five criteria for judging experimental animal infections as models for phage therapy of chronic infections as encountered in the clinic. As the first criterion should be assumed as a given, I start the list at zero:

0. Substantial delays, e.g., multiple days, weeks, or longer, following bacterial challenge; minimally including demonstration of some degree of infection stabilization prior to the onset of phage treatment.
1. Equivalent degrees of site preparation as used clinically prior to the onset of treatment, e.g., debridement in the case of wound infections.

Generally these should be followed with at least two of the following:

2. Demonstration, in most cases, of a *lack* of adequate treatment success without multiple, temporally separated phage applications over relatively long periods, i.e., if multiple doses over weeks are required in the clinic to adequately combat chronic infections then success using only a single dose in the laboratory ought to be suspect.
3. Demonstration, in many cases, of development of biofilms prior to treatment.
4. Demonstration of physiological development, by model infections, of antibiotic tolerance.

The latter point in a sense is an operational definition since chronic infections can become eligible for phage therapy due to a failure to be effectively treated using antibiotics—failures which are not necessarily consequences of genetically acquired resistance and particularly as associated with biofilm formation. Thus, chronic infection models for phage therapy in at least some cases should be ones for which antibiotics, as normally employed, have lost their ability to clear otherwise laboratory-sensitive bacteria over the course of infection development.

In conclusion, with few quibbles, I want to stress that I am delighted with Kishor et al.'s efforts. But, as with good science generally, as many questions are raised by their study as have been answered.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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The author has consulted and served on advisory boards for companies with phage therapy interests, holds equity stake in a number of these companies, and maintains the websites phage.org and phage-therapy.org. The text presented, however, represents the perspective of the author alone and no help was received in its writing.

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ORIGINAL RESEARCH
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Development of a Phage Cocktail to Control *Proteus mirabilis* Catheter-associated Urinary Tract Infections

*Luís D. R. Melo[1], Patrícia Veiga[1], Nuno Cerca[1], Andrew M. Kropinski[2], Carina Almeida[1], Joana Azeredo[1] and Sanna Sillankorva[1]**

[1] Laboratório de Investigação em Biofilmes Rosário Oliveira, Centre of Biological Engineering, University of Minho Braga, Braga, Portugal, [2] Departments of Food Science, Molecular and Cellular Biology, and Pathobiology, University of Guelph, Guelph, ON, Canada

Proteus mirabilis is an enterobacterium that causes catheter-associated urinary tract infections (CAUTIs) due to its ability to colonize and form crystalline biofilms on the catheters surface. CAUTIs are very difficult to treat, since biofilm structures are highly tolerant to antibiotics. Phages have been used widely to control a diversity of bacterial species, however, a limited number of phages for *P. mirabilis* have been isolated and studied. Here we report the isolation of two novel virulent phages, the podovirus vB_PmiP_5460 and the myovirus vB_PmiM_5461, which are able to target, respectively, 16 of the 26 and all the *Proteus* strains tested in this study. Both phages have been characterized thoroughly and sequencing data revealed no traces of genes associated with lysogeny. To further evaluate the phages' ability to prevent catheter's colonization by *Proteus*, the phages adherence to silicone surfaces was assessed. Further tests in phage-coated catheters using a dynamic biofilm model simulating CAUTIs, have shown a significant reduction of *P. mirabilis* biofilm formation up to 168 h of catheterization. These results highlight the potential usefulness of the two isolated phages for the prevention of surface colonization by this bacterium.

Keywords: bacteriophages, bacteriophage therapy, biofilms, urinary tract infection, *Proteus mirabilis*, phage cocktail

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Edited by:
Peter Mullany, UCL, UK

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Victor Krylov, Mechnikov Research Institute of Vaccines and Sera, Russia
Zuzanna Drulis-Kawa, University of Wroclaw, Poland

****Correspondence:***
Sanna Sillankorva
s.sillankorva@deb.uminho.pt

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INTRODUCTION

Indwelling urinary catheters are medical devices used by millions of people to relieve urinary retention and urinary incontinence. It has been estimated that more than 100 million urethral catheters are fitted each year in a range of healthcare facilities (Getliffe and Newton, 2006). Despite the important benefits brought by the use of these devices, catheters provide a suitable surface for the colonization of microorganisms and may further place at risk the patients' health, due to infections. In USA, catheter-associated urinary tract infections (CAUTIs) account for up to 40% of hospital-acquired infections (Saint et al., 2008). Also, 70% of urinary tract infections (UTIs) are associated with urinary catheters (Burton et al., 2011; Weber et al., 2011) and approximately 20% patients will suffer a catheterization during their hospital stay, especially in intensive care units (Saint and Lipsky, 1999).

The duration of the catheterization is a crucial risk factor of CAUTI development as almost all long-term catheterized (>28 days) patients develop a CAUTI, whereas in short-term (<7 days) catheterized patients only 10–50% develop an infection (Morris et al., 1999).

Proteus mirabilis is a leading cause of CAUTIs, being associated with up to 44% of all CAUTIs in the USA (O'Hara et al., 2000; Jacobsen et al., 2008). This microorganism, isolated from soil, stagnant water, sewage, and human intestinal tract, is associated with complicated infections, long-term catheterizations and urinary stone (struvite) formation (Armbruster and Mobley, 2012). *P. mirabilis* is a dimorphic bacterium that expresses thousands of flagella responsible for its swarming ability (Hoeniger, 1965). The virulence factors of *P. mirabilis* that contribute to the establishment of CAUTIs include the expression of fimbriae, that mediate the attachment to host cells and to catheters, and the consequent formation of dense biofilms on catheter surfaces (Armbruster and Mobley, 2012). Furthermore, it produces urease which is responsible for urea hydrolysis to carbon dioxide and ammonia that raises urine pH to above 8.3 (Stickler et al., 1998; Broomfield et al., 2009).

The microorganisms that colonize indwelling urinary catheters are commonly associated with antibiotic resistance and, thus, biofilm structures are frequently reported as reservoirs of antibiotic-resistant bacteria (Chenoweth et al., 2014). Although antibiotic therapy is successful in the majority of the cases, there has been a dramatic increase on antibiotic resistance among CAUTI-causing bacteria, including *P. mirabilis* (Wang et al., 2014). This fact makes it difficult to treat CAUTIs, highlighting the need for alternative preventive measures. During the last decades, the use of virulent bacteriophages (or phages) has re-emerged for therapeutic purposes (Viertel et al., 2014). After replicating inside the bacterial host, they cause cell lysis and release of phage progeny, which are able to infect neighboring cells. Phages are potential specific antibacterial agents, as they have self-replicating nature in the presence of the host cells, being eliminated from the human body in their absence (Azeredo and Sutherland, 2008). Furthermore, they are active against antibiotic-resistant bacteria, and phage preparations containing several phages, also known as phage cocktails, can be developed to increase their activity spectrum (Gu et al., 2012).

In the present study, two *P. mirabilis*-specific phages were isolated and characterized, and their effectiveness to control biofilm formation on silicone catheters was assessed in a dynamic biofilm model simulating CAUTIs.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A total of 26 *Proteus* spp. (18 *P. mirabilis*) strains were used in this study. Additionally, other members of the *Enterobacteriaceae* were used to assess the lytic spectra of the phages. The complete list of strains used in this study is provided in **Table 1**. Reference strains were obtained from *Salmonella* Genetic Stock Centre (SGSC) and Colección Española de Cultivos Tipo (CECT), while isolates were obtained from Laboratório de Análises Clínicas S. Lázaro (Braga, Portugal), from urine samples. Species was identified using selective agar media and biochemical tests. Bacteria were grown at 37°C in Tryptic Soy Broth (TSB; Liofilchem), on Tryptic Soy Agar (TSA; 1.5% agar) or in Artificial Urine (AU) (Brooks and Keevil, 1997) supplemented with 0.3% glucose (Silva et al., 2010).

Phage Isolation, Production, and Titration

Bacteriophage isolation was performed using the enrichment procedure, essentially as described before (Melo et al., 2014b), using raw sewage (Braga). Briefly, 50 mL of centrifuged effluent was mixed with the same volume of double-strength TSB and then inoculated with thirteen *P. mirabilis* strains (labeled with an "a" in **Table 1**). Fifty micro liter of each exponentially grown *P. mirabilis* culture were used. This solution was incubated for 18 h at 37°C, 120 rpm, centrifuged (10 min, 10,000 × *g*, 4°C) and the supernatant filtered through a 0.22 μm polyethersulfone membrane (GVS – Filter Technology). The presence of phages was checked by performing spot assays on bacterial lawns. Inhibition zones were purified to isolate all different phages on the respective bacterial host. Plaque picking was repeated until single-plaque morphology was observed and ten plaques of each isolated phage were measured and characterized.

Phage particles were produced using the plate lysis and elution method as described previously (Sambrook and Russell, 2001) with some modifications. Briefly, 10 μL phage suspension was spread on host bacterial lawns using a paper strip and incubated for 14–16 h at 37°C. After, 3 mL of SM Buffer [100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris/HCl (pH 7.5), 0.002% (w/v) gelatin] were added to each plate and incubated for 8 h (120 rpm on a PSU-10i Orbital Shaker (BIOSAN), 4°C). Subsequently, the liquid and top-agar were collected, centrifuged (10 min, 10,000 × *g*, 4°C). The lysate was further concentrated with PEG 8000 and then purified with chloroform and stored at 4°C.

Phage titration was performed according to the double agar overlay technique (Kropinski et al., 2009). Briefly, 100 μl of diluted phage solution, 100 μl of host bacteria culture, and 3 mL of soft agar were poured onto a Petri plate containing a thin layer of TSA. After overnight incubation at 37°C, the plaque forming units (PFUs) were determined.

Lytic Spectra of the Isolated Phages

The host range of the two isolated phages was determined by pipetting 10 μl of diluted phage solution (10^8 PFU.mL^{-1}) on lawns of indicated bacterial strains (**Table 1**). Plates were incubated 12 h at 37°C, and the presence and absence of inhibition zones indicating host sensitivity were reported.

Electron Microscopy

The morphology of phage particles was observed by transmission electron microscopy, as previously described (Melo et al., 2014b). Briefly, phage particles were collected after centrifugation (1h, 25,000 × *g*, 4°C). The pellet was washed twice in tap water using the same centrifugation conditions. Phages were further deposited on copper grids with carbon-coated Formvar

TABLE 1 | Phages lytic spectra on the bacterial strains used in this study.

Species	Strain	Source	Infectivity Pm5460	Infectivity Pm5461
Proteus mirabilis	ATCC 29906[a]	Culture collection	+	+
	ATCC 14153[a]	Culture collection	+	+
	SGSC 5460[a]	Culture collection	+	+
	SGSC 5461[a]	Culture collection	–	+
	933[a]	Human urine isolate (Braga)	–	+
	SGSC 3360[a]	Culture collection	–	+
	SGSC 5446[a]	Culture collection	+	+
	SGSC 5447[a]	Culture collection	+	+
	SGSC 5445[a]	Culture collection	–	+
	SGSC 5448[a]	Culture collection	+	+
	SGSC 5449[a]	Culture collection	+	+
	SGSC 5450[a]	Culture collection	–	+
	2380	Human urine isolate (Braga)	+	+
	2388B	Human urine isolate (Braga)	+	+
	9229	Human urine isolate (Braga)	–	+
	SLaz1[a]	Human urine isolate (Braga)	+	+
	SLaz2	Human urine isolate (Braga)	+	+
	SLaz3	Human urine isolate (Braga)	+	+
Proteus vulgaris	ATCC 6380	Culture collection	–	+
	ATCC 6896	Culture collection	–	+
	ATCC 13315	Culture collection	+	+
	ATCC 29905	Culture collection	–	+
	SGSC 3359	Culture collection	+	+
	SGSC 5469	Culture collection	+	+
Proteus hauseri	ATCC 13315	Culture collection	–	+
Proteus penneri	ATCC 33519	Culture collection	+	+
Citrobacter freundii	SGSC 5345	Culture collection	–	–
Citrobacter koseri	CK18	Human pus isolate (Braga)	–	–
Cronobacter sakazakii	ATCC 29544	Culture collection	–	–
Enterobacter aerogenes	ATCC 13048	Culture collection	–	–
Escherichia coli	ATCC 11775	Culture collection	–	–
	CECT 434	Culture collection	–	–
	Ec7	Human urine isolate (Braga)	–	–
	Ec8	Human urine isolate (Braga)	–	–
	Ec9	Human urine isolate (Braga)	–	–
Escherichia hermannii	ATCC 33650	Culture collection	–	–
Klebsiella pneumoniae	ATCC 11296	Culture collection	–	–
Morganella morganii	SGSC 5703	Culture collection	–	–
	M12	Human urine isolate (Braga)	–	–
Providencia rettgeri	R1	Human sputum isolate (Braga)	–	–
Providencia stuartii	S7	Human urine isolate (Braga)	–	–
Salmonella Enteritidis	ATCC 13076	Culture collection	–	–
Salmonella Typhimurium	ATCC 43971	Culture collection	–	–

[a]*strain used on the enrichment procedure for phage isolation.*

films, stained with 2% uranyl acetate (pH 4.0). Phages were observed using a Philips EM 300 electron microscope, and magnification was monitored with T4 phage tails (Ackermann, 2009).

One-step Growth Curve

One-step growth curve studies were performed as described previously (Rahman et al., 2011), with some modifications. Briefly, 10 mL mid-exponential-phase culture, OD_{620} 0.5, was harvested by centrifugation (5 min, 7000 × *g*, 4°C) and resuspended in 5 mL fresh TSB medium in order to obtain an OD_{620} of 1.0. The same volume of phage solution was added in order to achieve a multiplicity of infection (MOI) of 0.005. After adsorption during 5 min (37°C, 120 rpm) the mixture was centrifuged as described above, and the pellet resuspended in 10 mL of fresh TSB. Samples were taken every 5 min over a period of 30 min and every 10 min until 1 h of infection.

DNA Isolation, Genome Sequencing, and Annotation

Phage DNA was extracted as described before (Melo et al., 2014a). Purified phages were treated with 0.016% (v/v) L1 buffer [300 mM NaCl, 100 mM Tris/HCl (pH 7.5), 10 mM EDTA, 0.2 mg BSA mL^{-1}, 20 mg RNase A mL^{-1} (Sigma), 6 mg DNase I mL^{-1} (Sigma)] for 2 h at 37°C. After a thermal inactivation of the enzymes for 30 min at 65°C, 50 μg proteinase K ml^{-1}, 20 mM EDTA, and 1% SDS were added and proteins were digested for 18 h at 56°C. This was followed by phenol:chloroform:isoamyl alcohol solution (25:24:1, v/v) and chloroform extractions. DNA was precipitated with isopropanol and 3 M sodium acetate (pH 4.6), centrifuged (15 min, 7,600 × *g*, 4°C), and the pellet air-dried and further resuspended in nuclease-free water (Cleaver Scientific). Genome sequencing was performed on a 454 sequencing platform (Plate-forme d′ Analyses Génomiques at Laval University, Québec city, QC, Canada) to 50-fold coverage. Sequence data was assembled using SeqMan NGen4 software (DNASTAR, Madison, WI, USA). Phage genomes were autoannotated, using MyRAST (Aziz et al., 2008) and the presence of non-annotated CDSs, along with genes in which the initiation codon was miscalled, were checked manually using Geneious 6.1.6 (Biomatters). Potential frameshifts were checked with BLASTX (Altschul et al., 1997) and BLASTP was used to check for homologous proteins (Altschul et al., 1990), with an E value threshold of $<1 \times 10^{-5}$ and at least 80% query. Pfam (Finn et al., 2014) was used for protein motif search, with the same cutoff parameters as used with BLASTP. Protein parameters (molecular weight and isoelectric point) were determined using ExPASy Compute pI/Mw (Wilkins et al., 1999). The presence of transmembrane domains was checked using TMHMM (Kall and Sonnhammer, 2002) and Phobius (Kall et al., 2004), and membrane proteins were annotated when both tools were in concordance. The search of tRNA encoding genes was performed using ARAGORN (Laslett and Canback, 2004) and tRNAscan-SE (Schattner et al., 2005). Fragments 100 bp upstream of each predicted ORF were extracted and MEME (Bailey et al., 2009) was used to search for putative promoter regions that were further manually verified. For the same purposes, PHIRE was also used (Lavigne et al., 2004). ARNold (Naville et al., 2011) was used to predict rho-independent terminators and the energy was calculated using Mfold (Zuker, 2003). EMBOSS Stretcher (Rice et al., 2000) and CoreGenes (Zafar et al., 2002) were used for whole genome comparisons between *P. mirabilis* phages and their closest relatives. Phage 5460 was compared with Enterobacteria phages: K1-5 (NC_008152), UAB_Phi78 (NC_020414), and SP6 (AY370673). Phage 5461 comparisons were performed with *Yersinia* phage phiR1-RT (HE956709), and *Salmonella* phages STP4-a (KJ000058) and S16 (HQ331142). For phylogenetic analysis, homologous proteins were identified using BLASTP against the virus database at NCBI. A phylogenetic tree was constructed using "One Click" phylogeny.fr[1] (Dereeper et al., 2008). The data was exported in Newick tree format and opened in FigTree[2].

Evaluation of the Degree and Stability of Phage Coating

Silicone coupons were placed in 24-well plates and 1.7 mL of both phage solutions with different concentrations (10^6, 10^7, 10^8, 10^9, and 10^{10} PFU.mL^{-1}) were added. Plates were incubated overnight at room temperature without agitation. Coupons were removed, washed with SM Buffer and placed in a new well with the same volume SM Buffer.

Different sonication conditions were optimized to assure the efficient removal of viral particles without compromising the particles viability. Accordingly, a loss of viability for both phages was observed with an increase of the sonication period (>5 s) and amplitude (loss of viability at 25% > loss of viability at 22%) (Supplementary Figure S1). While it seems that amplitude increase has a negative effect on phage viability, there were no statistical differences between the 22 and 25% of amplitude ($p > 0.05$). Statistical differences were only noticed between the different sonication times for phage 5461 (for both amplitudes) and for phage 5460 (only for 25% of amplitude) ($p < 0.05$). Consequently, coupons were further sonicated for 5 sec at 22% amplitude and PFU.mL^{-1} were determined. The sonication was optimized to assure the efficient removal of viral particles without compromising virus viability using two different amplitudes (22 and 25%, Sonics Vibra-Cell VC 505 – VC 750 sonicator) during different sonication times (5, 10, and 20 s).

To further evaluate the "natural" release of phages from the silicone surface overtime, phage-coated coupons, were exposed to AU supplemented with 0.3% glucose at 37°C. For the surface coating, the concentration of the initial suspension was adjusted to obtain a concentration of adhered phages ranging from 10^5 to 10^6 phages/cm^2. At specific time points (2, 4, 6, 24, 48, 72, and 96 h) the number of released phages was determined by plaque assay.

Screening of *P. mirabilis* Strains for Biofilm Formation Ability

The stability of the phages was assessed after incubating the phages on AU at 37°C for 168 h with phages samples titration every 24 h.

For biofilm formation assays, 15 *P. mirabilis* strains (**Table 1**) were grown in 10 mL of AU supplemented with 0.3% glucose, and incubated for 16–18 h (orbital shaker ES-20/60 (BIOSAN), 120 rpm, 37°C). Bacterial cultures were centrifuged (3 min, 5,000 × *g*) and pellets resuspended in fresh AU to an OD_{620nm} of 0.1 (∼1×10^8 CFU.mL^{-1}). Twenty μl of each culture were added to 180 μl of fresh AU in a 96-well plate ($n = 6$) and were incubated for 48 h, at the conditions described above, with media renewal after 24 h. Negative controls were performed with 200 μl of AU ($n = 6$).

Biofilm biomass was quantified as previously described (Pires et al., 2011), with some modifications. Briefly, after 48 h of

[1]http://phylogeny.lirmm.fr/phylo_cgi/simple_phylogeny.cgi

[2]http://tree.bio.ed.ac.uk/software/figtree/

incubation, all media was removed and the wells washed twice with phosphate buffered saline (PBS), pH 7.5. After PBS removal, 220 µl of methanol were added for 20 min, the methanol was removed, and the plates were air-dried. Then, 220 µL of 1% crystal violet (w/v, Merck) were added to each well, incubated for 10 min at room temperature, and washed with tap water. Finally, 220 µL of 33% acetic acid (v/v, Fisher) were added to each well to dissolve the stain and the absorbance measured at 570 nm, in an ELISA reader (Tecan). Three independent experiments were performed. Strains were classified regarding biofilm formation, as previously described (Stepanovic et al., 2000). A cut-off (OD_c) was defined with three standard deviations above negative controls (AU). Four classes were defined: $OD \leq OD_c$ – non-biofilm forming strain; $OD_c < OD \leq 2 \times OD_c$ – weak biofilm forming strain; $2 \times OD_c < OD \leq 4 \times OD_c$ – moderate biofilm forming strain; $4 \times OD_c < OD$ – strong biofilm forming strain.

Efficacy of a Phage Cocktail under Dynamic Biofilm Formation Conditions

To mimic catheter conditions, biofilms were formed in a continuous model on Foley catheters (Silkemed Uro-cath 2Way, Overpharma). The flow was set to 0.5 $mL.min^{-1}$ to mimic the actual average flow in a catheterized patient (Jones et al., 2005; Levering et al., 2014). With the exception of the catheter, all other tubing of the system was autoclaved (15 min, 121°C). Under sterile conditions and with the aid of a sterile scalpel the two ends of the Foley catheters were removed to allow its attachment to the other tubing of the system. A phage cocktail containing the same concentration of phages Pm5460 and Pm5461 was prepared. Three mL of the phage cocktail at 10^9 $PFU.mL^{-1}$ (to obtain a 10^6 $PFU.cm^{-2}$ coating) were added to each catheter, the catheter was sealed and phage binding to the catheter material allowed to occur overnight at room temperature under static conditions. On the following day, all non-bound phages were removed flowing the catheter with SM Buffer, the flow systems was mounted under sterile conditions, and then catheters were supplied with AU inoculated with 1×10^5 $CFU.mL^{-1}$ of both *P. mirabilis* SGSC 5446 and SGSC 5449 (previously grown for 16 h in 10 mL AU supplemented with 0.3% glucose, centrifuged (3 min, 5,000 × *g*) and suspended at the desired concentration). Phage-coated and non-coated catheters were supplied with a continuous flow of fresh urine with the bacterial suspension for 24 h, and after with AU for up to 168 h with no recirculation of the medium. At 48, 96, and 168 h, 2 cm of each catheter were removed: 1 cm for microscopy analysis, and 1 cm for CFU analysis. To determine the number of viable cells, samples were resuspended in 1.5 mL NaCl 0.9%, sonicated for 5 s at 22%, and plated in TSA plates. Four independent assays were performed. The sonication step was previously optimized to assure complete detachment with no loss of cellular viability (see Supplementary Figure S1). Briefly, to optimize the sonication conditions for the detachment of cells, 1 mL of a fresh inoculum of *P. mirabilis* set to an OD_{620nm} of 0.1 was added to each well of a 24-well plate and sonicated using two different amplitudes (22 and 25%, Sonics Vibra-Cell VC 505 – VC 750 sonicator) during different sonication times (5, 10, 15, 20, and 40 s). Viable cells at each time point and amplitude were determined. Three independent assays were performed. The best conditions were applied to biofilms samples, and efficient removal was confirmed by microscopy.

Microscopy Observations of the Biofilms

Epifluorescence observations: To assess the ability of phages to control the biofilms formed on silicone catheters, catheter sections were stained with DAPI. Briefly, 0.5 cm sections of phage-coated or non-coated catheters taken at each time points were stained with 100 µl (100 $\mu g.mL^{-1}$) of DAPI and placed in the dark for 10 min. The excess of dye was removed using absorbent paper, the catheter sections were placed on microscope slides and analyzed using an epifluorescence microscope (Olympus, Model BX51, Hamburg, Germany) equipped with a CCD camera (Olympus, Model DP72) with a filter-block for DAPI fluorescence (Ex 365–370; Barrier 400; Em LP 421).

Scanning electron microscopy (SEM) observations: SEM observations were performed on washed (PBS) catheter sections, which had been gradually dehydrated in absolute ethanol (Merck) solutions (15 min each in 10, 25, 40, 50, 70, 80, and 100% v/v). The catheter sections, kept in a desiccator until observed, were sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

Statistical Analysis

All graphs were generated using GraphPad Prism 5 software (GraphPad Software). Means and standard deviations (SD) were calculated. Statistical analysis was carried out by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni *post hoc* tests. Both tests were performed used using GraphPad software. Differences between samples were considered statistically different for *p*-values lower than 0.05.

Nucleotide Sequence Accession Numbers

The complete genome sequences of the two *P. mirabilis* phage isolates were submitted to the GenBank under the accession numbers KP890822 (vB_PmiP_5460) and KP890823 (vB_PmiM_5461).

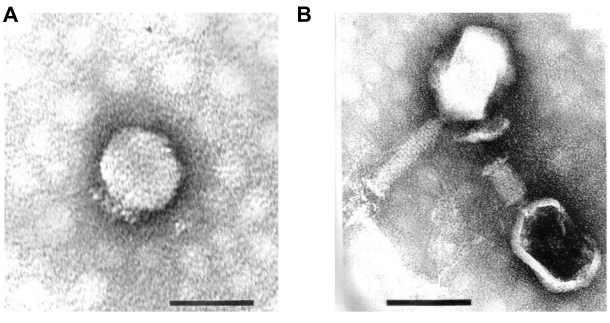

FIGURE 1 | Transmission electron micrographs of *Proteus mirabilis* phages: (A) Pm5460; (B) Pm5461. Scale bars represent 100 nm.

RESULTS

Isolation and Morphology of Proteus mirabilis Phages

These strains were used for phage enrichment (**Table 1**). In the initial screening, two different plaque morphotypes were detected. These plaques were purified and two different phages were isolated – vB_PmiP_5460 (5460) and vB_PmiM_5461 (5461), using *P. mirabilis* SGSC 5460 and SGSC 5461 as hosts, respectively. According to the morphological evaluation, phage 5460 belongs to the *Podoviridae* family of phages having a capsid 65 nm in diameter, a short (13 nm) tail terminating in tail fibers (**Figure 1A**). Phage 5461 has isometric head 87 nm in diameter, and a contractile tails (110 nm long and 17 nm wide) and belongs to the *Myoviridae* family (**Figure 1B**). Phage 5460 forms clear plaques 1.5 mm in diameter while phage 5461 forms smaller (1 mm) and less clear plaques.

Host Range Screening

To investigate the host specificity of both phages, a total of 43 strains of *Enterobacteriaceae* listed in **Table 1** were used. Phage 5460 lysed 12 out of 18 *P. mirabilis* strains (67%), three out of six *P. vulgaris* strains and one tested *P. penneri* strain; while phage 5461 killed all (100%) of the *Proteus* spp. tested. Notwithstanding the fact that only 43 strains were tested, both phages seem to have a *Proteus*-genus-specific profile, having no activity against other *Enterobacteriaceae* strains. It is likely that phage 5461 recognizes a conserved outer membrane protein (OMP) or polysaccharide such as the inner core region of lipopolysaccharide (LPS) which is known to be conserved among at least *P. mirabilis* strains (Vinogradov and Perry, 2000; Kaca et al., 2011).

One-step Growth Curve

The replication of phages 5460 and 5461 on their respective hosts (Supplementary Figure S2) revealed a latent and rise periods of phage 5460 of approximately 10 and 15 min, and an average burst size of 46 PFU per infected cell. On the other hand, phage 5461 showed a latency period of 25 min, a rise period of 10 min and a burst size of 11 PFU per infected cell. Within the 60 min of duration of the experiment, a second cycle of replication was evident for both phages.

Genome Analysis

Genome analysis revealed that both phages are virulent, not encoding any genes associated with lysogeny. Furthermore, no known virulence-associated or toxic proteins were detected *in silico*, revealing that both phages are potentially safe for therapeutic purposes.

Phage 5460 has a linear double-stranded DNA with 44,573 bp with a G+C content of 39.6% (**Table 2**). This phage encodes 53 putative CDSs, tightly packed occupying 93% of its genome. Of the CDSs, 20 have an assigned function and 11 are unique (Supplementary Table S1A). No tRNA genes were detected. The majority (90.6%) of the CDSs possess methionine as start codon, while only 5% a GTG start codon. BLASTN searches revealed that 5460 is homologous to the *Autographivirinae* phages: K1–5 (*Escherichia coli*), UAB_Phi78 (*Salmonella* Typhimurium) and SP6 (*Salmonella* Typhimurium). The comparison of the nucleotides of 5460 and these three phages with EMBOSS stretcher showed that 5460 has identities around 60% with these phages, being most similar (63.4%) to phage SP6 while CoreGenes (40) analysis showed that these phages share 33 homologs, which represent 62.3% of the 5460 proteins. Furthermore, the phylogenetic tree of the DNA polymerase containing homologs to 5460, demonstrated that this phage shares a branch with *Proteus* phages PM_85 and PM_93, being very close to the Enterobacteria phage SP6 (Supplementary Figure S3A). Consequently, this phage can be assigned to the *Sp6virus* genus.

Phage 5461 has a linear double-stranded DNA with 161,989 bp with a GC content of 31.1%, codifying 256 putative CDSs that occupy 95.2% of the genome (**Table 2**). From these CDSs, 123 have assigned function, while 90 are unique having no significant homologies to any proteins on public databases (Supplementary Table S1B). Ninety-five percent of the CDSs have an ATG as a start codon, while TTG (3%) and GTG (2%) are also used by this phage. This phage encodes eight different tRNA genes (tRNA-Glu, tRNA-Ser, tRNA-Asp, tRNA-Gly, tRNA-Pro, tRNA-Met, tRNA-Tyr, tRNA-Arg). BLASTN searches showed that the *Yersinia* phage phiR1-RT and *Salmonella* phages STP4-a and S16 are the closest relatives and therefore it can be assumed that this phage is part of the *Tevenvirinae* subfamily. EMBOSS stretcher alignment showed that 5461 and *Yersinia* phage phiR1-RT have an identity of 53.1%, while 5461 shares 52.2% of identity with *Salmonella* phages STP4-a and S16. CoreGenes (40) results showed that 5461 shares 133 homologous proteins with these three phages, which represent 45.3% of total proteins of 5461. The phylogenetic tree comprising the homologs of 5461 terminase large subunit shows that 5461 along with *Serratia* phage PS2 and *Citrobacter* phage Merlin are between the two ICTV approved taxa – *Js98virus* and *Cc31virus* (Supplementary Figure S3B).

Screening of *P. mirabilis* Strains for Biofilm Formation Ability

Phage stability in AU was assessed and both phages were shown to keep their titer after incubation in AU for 168 h (data not shown).

Then, an initial screening was performed to select strong biofilm-forming *P. mirabilis* strains according to the classification reported by Stepanovic et al. (2000). In total, biofilms of 15 different *P. mirabilis* strains were formed in AU for 48 h and the total biofilm biomass quantified (**Figure 2**). The vast majority of the strains were classified as moderate biofilm forming, five were defined as weak biofilm forming strains, *P. mirabilis* CECT 4101 was the only strain classified as a non-biofilm forming strain, and *P. mirabilis* strains SGSC 5449 and SGSC 5446 were the only strong biofilm-forming strains.

Evaluation of the Amount of Phages Coating the Silicone Surface

An efficient phage coating of the surfaces is critical to assure the success of an anti-biofilm strategy. Physical adsorption has been

TABLE 2 | *Proteus mirabilis* phages genome properties.

Phage	Genome size (Kb)	G+C content (mol%)	Putative CDSs	Promoters/terminators	tRNAs	Closest homolog (% identity)
Pm5460	44,573	39.6	56	10/5	0	Enterobacteria phage SP6 (63.4)
Pm5461	161,989	31.1	256	34/15	8	*Yersinia* phage phiR1-RT (53.1)

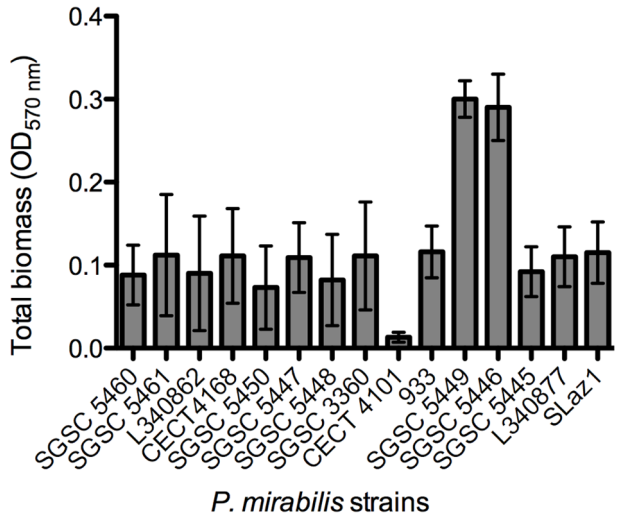

FIGURE 2 | Screening for biofilm formation of fifteen *P. mirabilis* isolates on 96-well microtiter plates using crystal violet staining. Data points represent an average of three independent experiments performed in triplicate. Error bars indicate standard deviation.

used to immobilize bacteriophages (Hosseinidoust et al., 2011), however, it cannot be assured a correct orientation of the phage (as tails must be available to interact with bacteria; Cademartiri et al., 2010). To better evaluate the effectiveness of phage physical adsorption, different phage concentrations were placed in contact with silicone surfaces (**Figure 3**). The amount of phages retained at the silicone surfaces increased proportionally to the initial concentration. The amount of 5460 phage particles adsorbed to the silicone surfaces was in average 1 log higher than the amount of 5461 phages (**Figure 3**).

Evaluation of Phage Release from Silicone Surfaces

The number of phages released from silicone surfaces was determined over time (**Figure 4**). The amount of phages 5460 released from the surfaces was in the range of 10^3 PFU.mL^{-1} (around 1% of the adhered phages). Phage 5461 was almost completely released from the surface after 2 h in contact with AU.

Dynamic Model for Biofilm Formation

The conditions found in real urinary catheters were mimicked using a continuous biofilm model system using Foley catheters. The viable cell counts and microscopy imaging of phage cocktail-coated catheters were compared with the control (non-phage coated) catheters (**Figure 5**). The higher concentration of biofilm cells obtained in each independent experiment was used to normalize the data.

During the assays, bacterial concentrations ranged between log 6 and log 8. According to the results, the phage-coating led to the reduction of the biofilm population and that difference was even more evident with the increase of biofilm age. Despite the non-significant differences of viable cells after 48 h between the

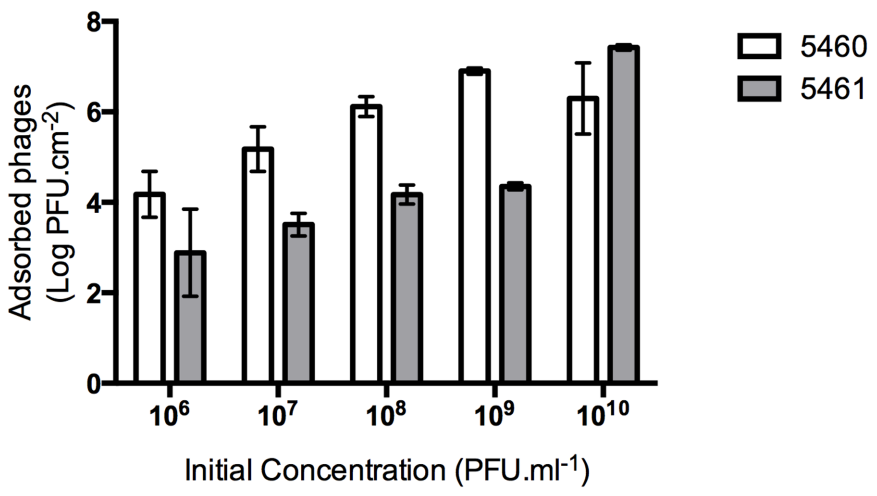

FIGURE 3 | Number of phages coating silicone surfaces per surface area. Data points represent an average of three independent experiments performed in triplicate and error bars indicate standard deviation. Statistical differences ($p < 0.05$) between the phage initial concentration and adsorbed phages were determined by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni *post hoc* test.

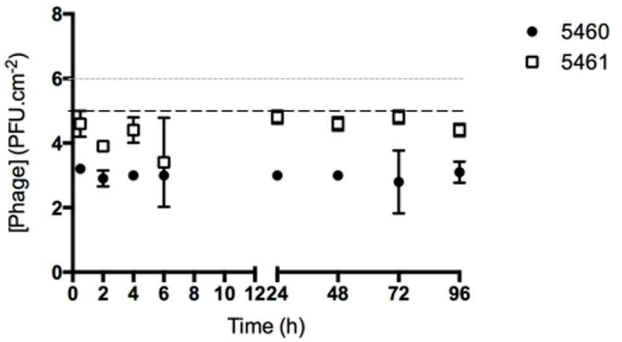

FIGURE 4 | Number of phages released from silicone surfaces per surface area. Data points represent an average of three independent experiments performed in triplicate and error bars indicate standard deviation. The approximate concentration of adhered 5460 phage is presented in the graphic as a black dot intermittent gray line, while approximate concentration of adhered 5461 phage is presented as an intermittent black line. Statistical differences ($p < 0.05$) between the approximate initial phage titer with released phage were determined by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni *post hoc* test.

phage-coated or non-coated catheters sections, a clear tendency of the phage cocktail to reduce *P. mirabilis* biofilms was already observable (**Figure 5**) leading to significant reductions ($p < 0.05$) at 96 and 168 h. This was also confirmed by epifluorescence and SEM (**Figure 5**) microscopy where more cells were observed in control catheter sections than in phage-coated catheters (**Figure 5**).

DISCUSSION

Urinary tract infections are involved in 40% of all nosocomial infections (Saint et al., 2008). Although Gram-positive bacteria, such as *Staphylococcus epidermidis* and *Enterococcus faecalis* can cause these infections, Gram-negative *Enterobacteriaceae*, are the most commonly implicated in CAUTI development (Siddiq and Darouiche, 2012). *P. mirabilis* forms crystalline biofilms within the urinary tract and is responsible for up to 30% of all urinary tract stones (struvite) (Stickler and Morgan, 2006). Furthermore, these crystalline structures recurrently block the flow through catheters (Stickler and Feneley, 2010). As frequently observed with different bacterial species, *P. mirabilis* strains are important reservoirs of antibiotic resistant determinants (Harada et al., 2014) and, because of this, resistant phenotypes have emerged in last years (Harada et al., 2014; Wang et al., 2014). Furthermore, as in other species, *P. mirabilis* biofilms were shown to be more tolerant to several antibiotics, than their planktonic counterparts (Moryl et al., 2013).

On the last decades the use of phages as alternatives or complements to antibiotic therapy has extensively been evaluated (Viertel et al., 2014) and has even been listed by the US National Institute of Allergy and Infectious Diseases as one important approach to combat antibiotic resistance (Reardon, 2014). The current relevance of phage therapy associated with the lack of *P. mirabilis* phages described so far, were the underlying reasons for this study.

Two *P. mirabilis* phages, isolated from raw effluents of wastewater treatment plants, were characterized. TEM analysis demonstrated that both belong to the *Caudovirales* order and are, respectively, members of the *Podoviridae* (phage 5460) and the *Myoviridae* (phage 5461) families. Their spectrum of activity against a collection of *Proteus* spp. was 16 out of 26 strains for phage 5460 and 26 out of 26 strains for 5461. Both phages were unable of killing other Enterobacteriaceae strains tested. Despite both phages have shown specificity to *Proteus* genera, it should be noted that only a limited number of strains of other species was used. As expected, these two phages had clear differences in the replication parameters, which resulted in higher burst size for the *Podoviridae* phage.

Based upon the genome sequence, phage 5460 belongs to the *Autographivirinae* subfamily, specifically to the *Sp6virus* genus. The GC content of phage 5460 is 39.6%, a value similar to *P. mirabilis* HI4320 strain (GC 38.9%) and to *Proteus* phage PM16 (41.4%G+C) and 5460 shares protein homology with enterobacterial *Podoviridae* phages K1-5 (63.5%), SP6 (63.5%), and UAB_Phi78 (56.9%).

Despite the morphological similarities with *Myoviridae* of the T4-like phages, phage 5461 has different characteristics from those deposited in GenBank. For instance, the GC content of 5461 is 31.1%, which is clearly lower than the content observed in other T4-like viruses and in its host species. This low GC content may cause codon usage problems during phage infection (Rocha and Danchin, 2002). The presence of eight tRNA genes on 5461 genome might attenuate these differences, as the presence of tRNAs in phage genomes is correlated with the differences in the codon usage between the phage and the host, corresponding to codons that are expected to be poorly translated by the host machinery (Bailly-Bechet et al., 2007). Phage 5461 shares homologous proteins with the myoviruses *Yersinia* phage phiR1-RT (44.3%) and *Salmonella* phages STP4-a (45.1%) and S16 (43.3%). Furthermore, the phylogenetic analysis have shown that this phage cannot be assigned to a known genus.

The two phages characterized were studied for their potential preventive activity toward biofilms. For this, silicone surfaces were coated with phages using standard adsorption procedure. The fact that phage 5460 adsorbed in greater amounts than phage 5461 can be due to the different morphological characteristics and composition of the phages. Hosseinidoust et al. (2011), described that the morphology of the phages has a great influence on phage adsorption due to their orientation on the surface. Podoviruses are shorter that myoviruses, therefore the ratio between the surface area and the phage length is greater for podophages, consequently it is expected that a greater quantity of 5460 are adsorbed to silicone surfaces.

In order to obtain a stable coating it is important that phages remained attached to the surface during flow (Lehman and Donlan, 2015), therefore phage release from surfaces was also evaluated by titer determination. The results pointed out to a greater adhesion strength between phage 5460 and the silicone surface compared to phage 5461. It has been previously shown

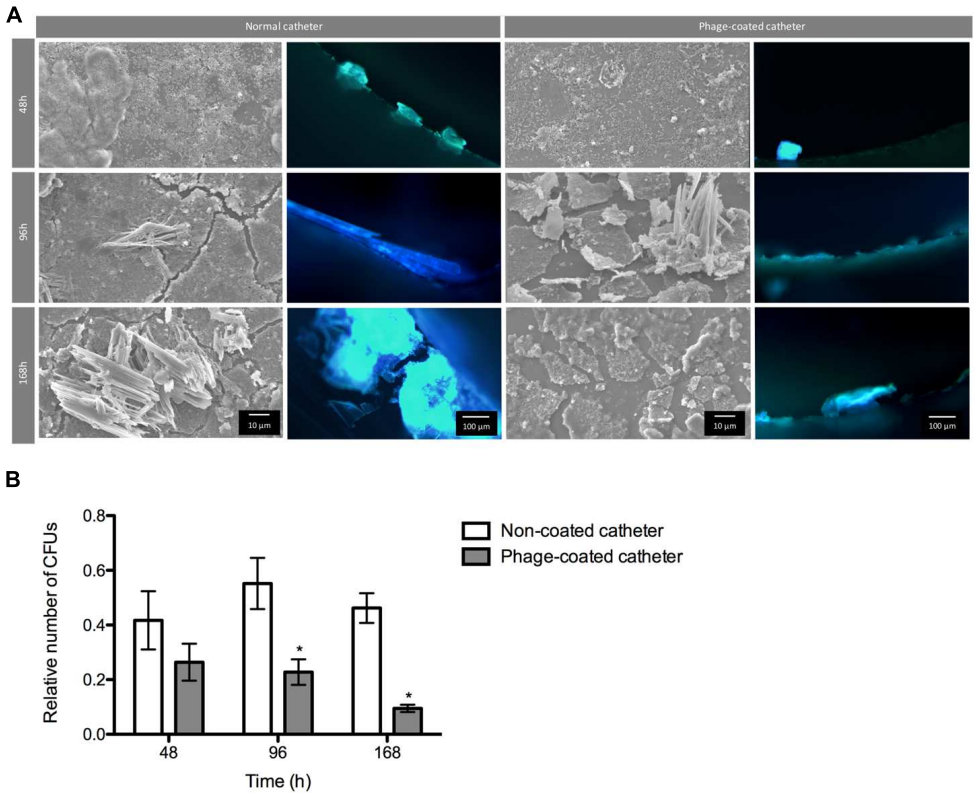

FIGURE 5 | Phage cocktail effect on *P. mirabilis* biofilms under dynamic conditions. Representative SEM and epifluorescence images **(A)** and relative number of CFU **(B)** of *P. mirabilis* biofilms formed in normal and phage-coated Foley catheter show a decrease on the biofilm population, as well as on the overall biomass. SEM images are not representative of the overall biofilm as they are focused on spaces where biofilm is present. Relative CFU values were normalized using the higher concentration of biofilm cells obtained in each independent experiment. Magnification of 100x and 1000x, were used for epifluorescence and SEM images, respectively. Data points represent an average of four independent experiments and error bars indicate standard deviation. Statistical differences ($p < 0.05$) between control biofilms and phage-cocktail- -treated biofilms (*) were determined by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni *post hoc* test.

that phages from different families have different surface-coating properties (Hosseinidoust et al., 2011). However, the reason why the coating stability differs between phages is not apparent as the phages major capsid proteins – the most abundant component of the phage particle - present similar isoelectric points (phage 5460: 5.15; phage 5461: 5.10) and hydrophibicities (phage 5460: −0.23; phage 5461: −0.33), which would suggest the same ability to interact with the surface. Although the majority of the adhered phages are released in short periods, the host can colonize the catheter surface forming biofilms. At this stage, not only the microbial cells adhered to the catheter surface, but also the biofilm by itself are phage reservoirs (Doolittle et al., 1996). Therefore, phages within the biofilm will be available to infect neighbor cells delaying biofilm formation and development.

After performing a rapid screening for biofilm formation in different *P. mirabilis* strains, two good biofilm-forming strains were selected for further tests in dynamic models. The two good biofilm-forming strains have shown similar susceptibly to both phages and thus have provided a suitable combination for the dynamic studies.

Due to their distinct lytic spectra and surface binding characteristics, a cocktail of both phages was used to prevent biofilm formation under dynamic conditions. Since urinary catheters can stay inserted in the patients' bladder for long periods, the efficacy of phage-coated catheters was prolonged up to 7 days. In fact, the phage cocktail developed herein reduced significantly the total number of cultivable cells after 96 and 168 h.

An important feature of *P. mirabilis* biofilms is its great ability to form crystals, which associated with bacterial cells attached to surfaces can lead to clogging of the catheter (Coker et al., 2000; Stickler et al., 2006). During our experiments this ability was evident as crystalline biofilms were seen microscopically and even by simple observation of the tubing at naked eye. Indeed, the difference in biofilm biomass in phage-coated or non-coated catheters were also apparent macroscopically.

Other authors have evaluated the use of a phage cocktail in the prevention and eradication of *E. coli* and *P. mirabilis* biofilms (Carson et al., 2010) and observed significant reductions (3 to 4 log) in the *E. coli* population, however, only a 1 log reduction was observed for the *P. mirabilis* population. These authors suggested

that this lower efficacy might be related with phage-dependent factors, such as the production of depolymerases and the ability to penetrate the EPS matrix. In the case of the phage cocktail developed herein, this can also be one of the reasons for the low reductions (1 log) observed for the *P. mirabilis* biofilm. More recently, Lehman and Donlan (2015) used a phage cocktail on the pre-treatment of hydrogen-coated silicone catheters to prevent the adhesion of *Pseudomonas aeruginosa* and *P. mirabilis*. After treatment, the authors detected a higher decrease on CFU counts of *P. aeruginosa* than of *P. mirabilis*. Nonetheless, reduction in the *P. mirabilis* population was still pronounced with a log reduction of approximately 2, for both single and mixed biofilms.

Other reasons that might be hampering phage efficacies might be physico-chemical factors such as pH and ions (Jonczyk et al., 2011) and also the fact that the adsorption of phages to their target cells may be inhibited by the crystals formed in AU during biofilm formation. Furthermore, the biofilm matrix itself can block the specific phage receptors, preventing phage infection from occurring (Samson et al., 2013). Finally, the physiological state of the cells could also be influencing phage efficacy, since log-phase cells are faster and more efficiently lysed than cells with lower metabolic rates, such as the ones that compose biofilms (Hadas et al., 1997).

The specificity of the phages, their genome features, which do not encode genes associated with lysogeny, together with the moderate reductions obtained using *in vitro* simulated conditions, support the use of both phages vB_PmiP_Pm5460 and vB_PmiM_Pm5461 for preventing *P. mirabilis* biofilm formation on silicone catheters.

AUTHOR CONTRIBUTIONS

NC, CA, JA, and SS conceived the study. AK, CA, JA, and SS analyzed data. LM and PV performed experiments. LM wrote the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Role of the Pre-neck Appendage Protein (Dpo7) from Phage vB_SepiS-phiIPLA7 as an Anti-biofilm Agent in Staphylococcal Species

*Diana Gutiérrez[1], Yves Briers[2,3], Lorena Rodríguez-Rubio[1,2], Beatriz Martínez[1], Ana Rodríguez[1], Rob Lavigne[2] and Pilar García[1]**

[1] Consejo Superior de Investigaciones Científicas – Instituto de Productos Lácteos de Asturias, Villaviciosa, Spain, [2] Laboratory of Gene Technology, KU Leuven, Heverlee, Belgium, [3] Laboratory of Applied Biotechnology, Ghent University, Ghent, Belgium

Staphylococcus epidermidis and *Staphylococcus aureus* are important causative agents of hospital-acquired infections and bacteremia, likely due to their ability to form biofilms. The production of a dense exopolysaccharide (EPS) matrix enclosing the cells slows the penetration of antibiotic down, resulting in therapy failure. The EPS depolymerase (Dpo7) derived from bacteriophage vB_SepiS-phiIPLA7, was overexpressed in *Escherichia coli* and characterized. A dose dependent but time independent response was observed after treatment of staphylococcal 24 h-biofilms with Dpo7. Maximum removal (>90%) of biofilm-attached cells was obtained with 0.15 μM of Dpo7 in all polysaccharide producer strains but Dpo7 failed to eliminate polysaccharide-independent biofilm formed by *S. aureus* V329. Moreover, the pre-treatment of polystyrene surfaces with Dpo7 reduced the biofilm biomass by 53–85% in the 67% of the tested strains. This study supports the use of phage-encoded EPS depolymerases to prevent and disperse staphylococcal biofilms, thereby making bacteria more susceptible to the action of antimicrobials.

Keywords: exopolysaccharide depolymerase, biofilm, biofilm matrix, *S. epidermidis*, *S. aureus*

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Brigham Young University, USA

****Correspondence:***
Pilar García
pgarcia@ipla.csic.es

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INTRODUCTION

Biofilms are surface-attached microbial communities surrounded by a polymeric matrix, which protects them from the external environment. This complex structure confers to the bacteria a high resistance to antibiotics, disinfectants and to the host, an immune system response (Hall-Stoodley and Stoodley, 2009). The ability to develop a biofilm is one of the major virulence factors in many pathogenic bacteria, while it is a key factor for colonization in opportunistic bacteria. *Staphylococcus epidermidis* is a prominent example of a bacterial species with a pathogenicity associated to the biofilm formation on medical devices (Rupp, 2014), being the most common cause of bacteremia in immune-compromised patients (Rogers et al., 2009). In addition, methicillin resistant strains (MRSE) carrying the staphylococcal cassette chromosome *mec* (SCC*mec*), are widely spread within hospitals (Iorio et al., 2012), and provide a reservoir of resistance that might be transferred to *Staphylococcus aureus* (Otto, 2013), also a frequent cause of nosocomial infections. *S. aureus* encodes a number of virulence factors that enable to infect host tissues (Lowy, 1998). In addition, methicillin-resistant *S. aureus* (MRSA) isolates, resistant to all available penicillins and other β-lactam antibiotics, have rapidly disseminated beyond clinical settings among general population (David and Daum, 2010) and livestock (Price et al., 2012).

Extracellular material of staphylococcal biofilms is a complex combination of polysaccharides, teichoic acids, proteins and DNA. The polysaccharide intercellular adhesin (PIA/PNAG) is a poly-β-1,6-*N*-acetyl glucosamine, which production is dependent on the presence of the operon icaADBC (Mack et al., 1996). However, staphylococcal strains with a PIA/PNAG-independent biofilm were also identified. In this case, the extracellular matrix is based on the presence of adhesive proteins such as Aap, Embp, Bhp, and Bap (Hussain et al., 1997; Cucarella et al., 2001; Zhang et al., 2003; Christner et al., 2010). The presence of teichoic acids in staphylococcal species enhances adhesion of bacterial cells to fibronectin, increasing their pathogenicity (Hussain et al., 2001; Vergara-Irigaray et al., 2008). In addition, a number of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are able to interact with the host matrix proteins (Patti et al., 1994). Finally, the modulation of the extracellular genomic DNA (eDNA) release and degradation was shown as an important factor in biofilm maturation (Mann et al., 2009).

Due to the importance of biofilms in hospital settings, several approaches to prevent and remove them have been assayed. New materials were designed by coating their surface with different antimicrobials, like bacteriocins and essential oils, which caused a reduction in biomass and viability of bacteria in biofilms (Nostro et al., 2010, 2013). Other changes in physicochemical properties of biomaterial surfaces resulted in reduction of bacterial adhesion and further biofilm formation (Tang et al., 2009). Additional strategies were focused on small molecules that interfere with the expression of virulence genes including those necessary for biofilm formation (Ma et al., 2012). Finally, it is noteworthy that previously formed biofilms can be removed by degrading the extracellular matrix or by killing the bacteria inside the structure. Alternatives to antibiotics for killing bacteria and biofilm removal include bacteriocins (Saising et al., 2012), bacteriophages (Cerca et al., 2007; Gutiérrez et al., 2012, 2015) and endolysins (Domenech et al., 2011; Shen et al., 2013; Gutiérrez et al., 2014). However, to eradicate the biofilm structure a successful degradation of extracellular matrix is required. Regarding this, PIA-degrading enzymes like dispersin B (Kaplan et al., 2004) and bacteriophage-encoded polysaccharide depolymerase proteins (Cornelissen et al., 2012) have been proposed.

We have previously identified and characterized two bacteriophages infecting *S. epidermidis*, vB_SepiS-phiIPLA5 and vB_SepiS-phiIPLA7, which exhibit plaques surrounded by an increasing halo zone indicative of the presence of polysaccharide depolymerase activity (Gutiérrez et al., 2010). Genomic characterization of phage vB_SepiS-phiIPLA7 showed a protein (gp18, 98.5 kDa) located in the structural region containing two catalytic domains. A putative pectin lyase domain was identified at the amino-terminal part of the protein, and a putative peptidase domain at the C-terminus. It was suggested that the anti-biofilm activity showed by phage vB_SepiS-phiIPLA7 may be attributed to this protein (Gutiérrez et al., 2012). In the present work, protein gp18 named Dpo7, was overexpressed in *E. coli* and purified. The polysaccharide depolymerase activity of Dpo7 was confirmed against *S. epidermidis* and *S. aureus* biofilms.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Ten different *S. epidermidis* strains and two *S. aureus* strains were used in this study (**Table 1**). All bacteria were isolated in Baird–Parker (BP) agar and routinely cultured in TSB broth (Tryptic Soy Broth, Scharlau, Barcelona, Spain) at 37°C with shaking or in TSB plates containing 2% (w/v) bacteriological agar (TSA). *E. coli* transformants were selected on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 2% (w/v) bacteriological agar and 100 μg ml^{-1} ampicillin at 37°C.

Cloning and Overexpression of Dpo7

The codon usage of the *orf18* gene, encoding Dpo7 (GenBank accession number YP_006561180.1) from phage vB_SepiS-phiIPLA7 was optimized based on *E. coli* codon usage by the OptimumGene^{TM} Codon Optimization Technology. Additionally, *Nde*I and *Xho*I restriction sites were added at the 5′ and 3′ ends of the sequence, respectively. The optimized sequence was synthesized and cloned into pUC57 vector by GenScript (Township, NJ, USA). Afterward, a *Nde*I-*Xho*I fragment containing the *orf18* gene was released from pUC57 and sub-cloned into pET21a(+) vector (EMD Biosciences, San Diego, CA, USA), which introduces a C-terminal 6 His-tag. The construct (pET21a-*dpo7*) was verified by DNA sequencing using vector-specific primers (T7 promoter: 5′-TAATACGACTCACTATAGGG-3′ and T7 terminator 5′-GCTAGTTATTGCTCAGCGG-3′, Eurogentec, Madrid, Spain) and two internal primers (5′-TCAGAAAGATTCCACGAAGG-3′ and 5′-TAATGGCCATGTGAGCATC-3′, Eurogentec, Madrid, Spain).

The plasmid pET21a-*dpo7* was electroporated in *E. coli* BL21 (DE3) pLysS (Invitrogen Corporation, Gent, Belgium) and protein expression was carried out as described previously (Obeso et al., 2008) with 1 mM of IPTG for 16 h at 16°C. Five hundred milliliter culture cells were pelleted, suspended in 10 ml lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, pH 7.4) and frozen/thawed three times at −80°C. Sonication was

TABLE 1 | Strains used in this study.

	Strain	Reference
S. epidermidis	F12	Delgado et al., 2009
	B	
	DH3LIK	
	YLIC13	
	Z2LDC14	
	DG2n	
	ASLD1	
	LO5081	
	LV5RB3	
	LO5RB1	
S. aureus	15981	Valle et al., 2003
	V329	Cucarella et al., 2001

carried out afterward 15 × 5 s pulses with 15 s recovery on ice and centrifuged at 10,000 × *g*. The supernatant containing the protein was purified using the His GraviTrap column kit (GE Healthcare Life Sciences, Buckinghamshire, UK) following the supplier's recommendations. Wash buffer and lysis buffer were composed of 20 mM NaH_2PO_4, 500 mM of NaCl, pH 7.4 with 50 or 500 mM imidazole, respectively. Protein purity was estimated by SDS-PAGE and the predicted amino acid sequence was confirmed by mass spectrometry (MALDI-TOF/TOF) as previously described (García et al., 2004). Protein amount was quantified by the Quick Start Bradford Protein Assay (BioRad, Hercules, CA, USA). Prior to the activity assays, the purified Dpo7 was dialyzed for 16 h against PBS buffer (137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4; pH 7.4).

Biofilm Assays

Biofilms of staphylococcal strains were grown into a TC Microwell 96U w/lid nunclon D SI plates (Thermo Scientific, NUNC, Madrid, Spain). Overnight cultures were diluted in TSBg [TSB supplemented with 0.25% w/v D-(+)-glucose] up to 10^6 CFU/ml and 200 μl were poured into each well and incubated for 24 h at 37°C. Wells were washed twice with sterile phosphate-buffered saline (PBS buffer) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4; pH 7.4). After treatment of biofilms (see below), cultivable bacteria released in the supernatant were counted by plating on TSA serial dilutions. Moreover, cultivable adhered bacteria were determined by scratching twice with a sterile swab and then immersed into 9 ml of PBS buffer followed by a vigorous shaking for 1 min. Finally, several decimal dilutions were plated onto TSA and incubated at 37°C. Biomass quantification of biofilm adhered to the surface of wells was carried out as previously described (O'Neill et al., 2008). Briefly, wells were treated with crystal violet (0.1% w/v) for 15 min, followed by a gentle wash with water and de-staining in acetic acid (33% v/v). Finally, absorbance was measured at 595 nm. To determine the matrix composition, the biofilms were washed with PBS and then treated for 1 h at 37°C with a solution of 10 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.5) (to disrupt the extracellular polysaccharides), with 100 mg/ml of proteinase K (Sigma, Madrid, Spain) in 20 mM Tris HCl (pH 7.5) and 100 mM NaCl, or with 100 mg/ml of DNaseI (Sigma, Madrid, Spain) in 150 mM of NaCl and 1 mM $CaCl_2$ (Kaplan et al., 2004; Holland et al., 2011). After treatments, the biofilms were washed with water, stained with crystal violet, and the absorbance measured as described above. All assays were performed using four biological replicates.

Characterization of Dpo7 Activity

To determine the optimal conditions for Dpo7 activity, different concentrations of the protein (0–1.5 μM) or PBS buffer for control purposes, were added to 24 h-preformed biofilms of *S. epidermidis* F12, and incubated for different times (30 min – 24 h) at temperatures ranging from 22 to 37°C. Biofilm removal was quantified by enumeration of cultivable bacteria and crystal violet staining (see above).

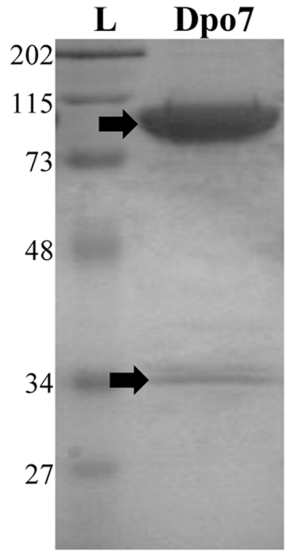

FIGURE 1 | Purification of the recombinant exopolysaccharide depolymerase Dpo7 from *Escherichia coli* BL21(DE3) pLys pET21a-*dpo7*. L: Standard molecular weight marker in kDa (Low Range Molecular Weight SDS-PAGE Standards, BioRad); Fraction eluted from nickel affinity chromatography containing purified Dpo7. Bands indicated with a black arrow were identified by mass spectrometry.

The activity of the protein was also tested against the biofilms formed by other staphylococcal strains using 0.15 μM of Dpo7 for 3 h at 37°C. All experiments were performed in triplicate.

The ability of Dpo7 to prevent biofilm formation was also tested using a conventional broth microdilution technique. Two-fold dilutions of Dpo7 (0–1.5 μM) in TSBg were added to TC Microwell 96U w/lid nunclon D SI plates (Thermo Scientific, NUNC, Madrid, Spain) in order to test biofilm formation and to 96-Well Microtiter™ Microplates (Thermo Scientific, NUNC, Madrid, Spain) to assess planktonic bacterial growth. Each well was inoculated with 10^6 CFU/well of bacteria. Plates were incubated at 37°C for 24 h. Biofilm formation was quantified by crystal violet staining and measuring the absorbance at 595 nm. Planktonic growth was determined by measuring the absorbance at 600 nm of each supernatant.

Dpo7 activity against extracellular material was assessed by using exponential cultures (OD_{600} = 0.6) of *S. epidermidis* F12 in TSBg. Cells were suspended in PBS buffer containing 0.15 μM of Dpo7 and incubated for 3 h at 37°C. Three microliter of the cells diluted in 10 μl of 1% Congo red aqueous solution (Sigma–Aldrich, St. Louis, MO, USA) were spread onto a glass slide and air-dried. To visualize the extracellular material, staining with Maneval's solution was performed as previously described (Cornelissen et al., 2011).

Quantification of lytic activity of Dpo7 was tested against live *S. epidermidis* F12 cells prepared as previously described (Becker et al., 2009), using the turbidity reduction assay (Obeso et al., 2008).

The pH stability of the protein Dpo7 was tested by dilution (1.5 μM) into the Britton–Robinson pH universal buffer

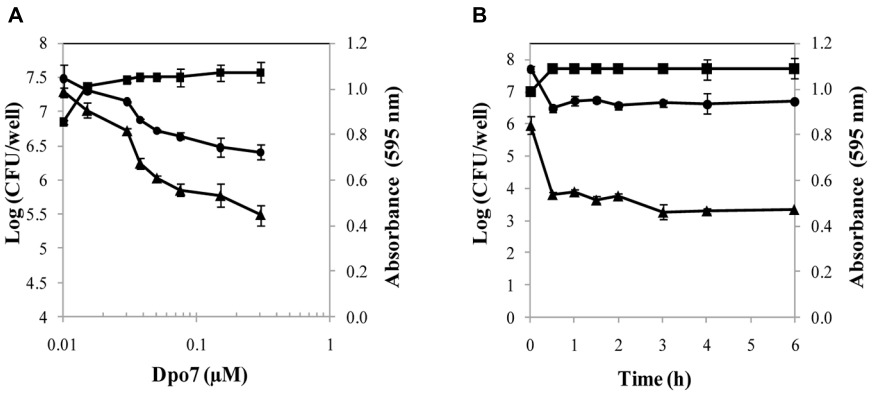

FIGURE 2 | Activity of Dpo7 against 24 h-biofilms of *Staphylococcus epidermidis* F12 in PBS buffer at 37°C. Biofilm disruption was determined using **(A)** different concentrations of Dpo7 (μM) for 3 h at 37°C and **(B)** 0.15 μM of Dpo7 throughout time ranging 30 min to 6 h. Total attached biomass was measured by crystal violet staining after treatment and expressed as A_{595} units (▲); Adhered cultivable bacteria (●) and supernatant cultivable bacteria (■) are expressed as Log (CFU/well). Each value corresponds to the mean ± standard deviation of three independent experiments.

(150 mM KCl, 10 mM KH_2PO_4, 10 mM sodium citrate, 10 mM H_3BO_3, adjusted within a pH 3–11 range, and subsequent maintenance at room temperature for 1 h. The protein was then diluted 10-fold in PBS buffer. For control purposes, Britton–Robinson buffer was diluted 10-fold in PBS buffer. Similarly, the temperature stability was examined after incubation of 0.15 μM of Dpo7 in PBS buffer for 30 min at different temperatures (ranging from 40 to 90°C). The activity of the protein after these treatments was tested against 24 h-biofilms of *S. epidermidis* F12 as indicated above.

Statistical Analysis

A one-way analysis of variance (ANOVA) and the LSD test was carried out to establish any significant differences regarding the adhered cells and cells number in the supernatant and biomass between the control and the treated biofilms. The differences were expressed as the mean ± standard error and the level of significance was established at $P < 0.05$ (SPSS11.0 Software for windows; Chicago, IL, USA).

RESULTS

Dpo7 is Able to Remove *S. epidermidis* Extracellular Material in Both Biofilms and Planktonic Cells

To determine the activity of the recombinant protein Dpo7, a synthetic gene with optimized codons was used for expression in *E. coli* BL21 (DE3) pLysS as an N-terminal 6×-His-tagged fusion, allowing purification by immobilized metal chelate affinity chromatography. The purity of the protein was estimated to be 95% as assessed by SDS-PAGE analysis (**Figure 1**). A main band of about 98 kDa corresponding with the molecular weight of Dpo7 was observed. A minor additional band (32 kDa) was identified by mass-spectrometry as a degradation product of Dpo7. Stocks of about 15 μM of protein were routinely obtained in these conditions.

The activity of the recombinant Dpo7 was characterized by treatment of 24 h-biofilms of the strain *S. epidermidis* F12 with different concentrations of Dpo7 for 3 h at 37°C (**Figure 2A**). Total biomass that remained attached after the Dpo7 treatment was quantified by crystal violet staining. The maximum biofilm disruption (43%) was obtained with 0.075 μM and higher concentrations (up to 1.5 μM) did not improve significantly this result ($P < 0.05$). Moreover, the number of cultivable bacteria was also determined after Dpo7 treatment. The maximum removal of attached cells (about 1 log unit; 92% of the total) was obtained treating the biofilms with 0.15 μM of the recombinant protein. This value correlates with the increase in the number of cells in the supernatant (**Figure 2A**) but the total cell number (adhered plus planktonic) remained constant (data not shown), indicating that biofilm-associated cells were released to the planktonic state without any lytic activity of Dpo7. A turbidity reduction assay confirmed the absence of lytic activity in Dpo7. There were no statistical differences between the control and the treated cells (data not shown). These results support the hypothesis that the activity of Dpo7 is related with the degradation of the extracellular material that surrounds the bacteria inside the biofilm rather than lysis of the cells.

Once the Dpo7 minimum concentration to disrupt 50% of the biofilm was established, an assay with different time exposures was carried out at 37°C (**Figure 2B**). For control purposes, the number of cultivable bacteria and biomass staining were recorded in biofilms treated only with PBS buffer for each time, but no differences were observed during the assay. The results showed that the protein was active and most of biofilm matrix degradation took place within the first 30 min. The minimum adhered cells and the maximum cells in the supernatants were also detected 30 min after treatment, remaining stable until the end of the exposure (**Figure 2B**). Longer times of incubation (until 24 h) did not change these results.

The optimum parameters for Dpo7 activity were determined by incubation at several temperatures (22, 32, and 37°C). Dpo7 was active in all temperatures tested with increasing activity at

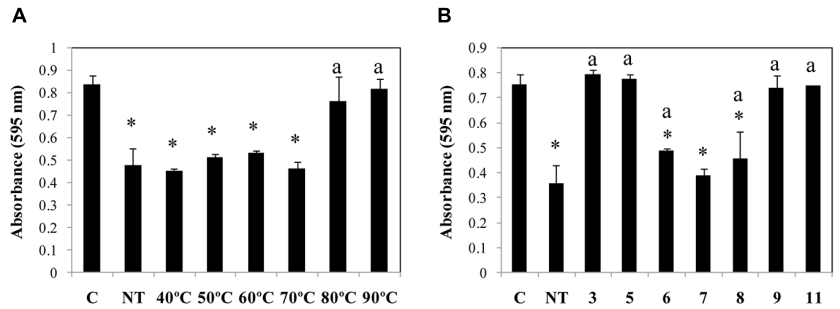

FIGURE 3 | Temperature (A) and pH (B) stability of Dpo7. Temperature stability was tested by incubation of Dpo7 (0.15 μM) for 30 min and pH stability after maintenance at room temperature for 1 h. C: control biofilm of *S. epidermidis* F12 without Dpo7 treatment; NT: activity of Dpo7 at pH 7.4 without temperature treatment. Values represent the mean ± standard deviation of three independent experiments. Bars having an asterisk are statistically significant different from the control and bars with a lower case 'a' indicate a statistically significant difference between the biofilm treatment with standard Dpo7 and the activity after thermal or pH treatment (ANOVA; $P < 0.05$).

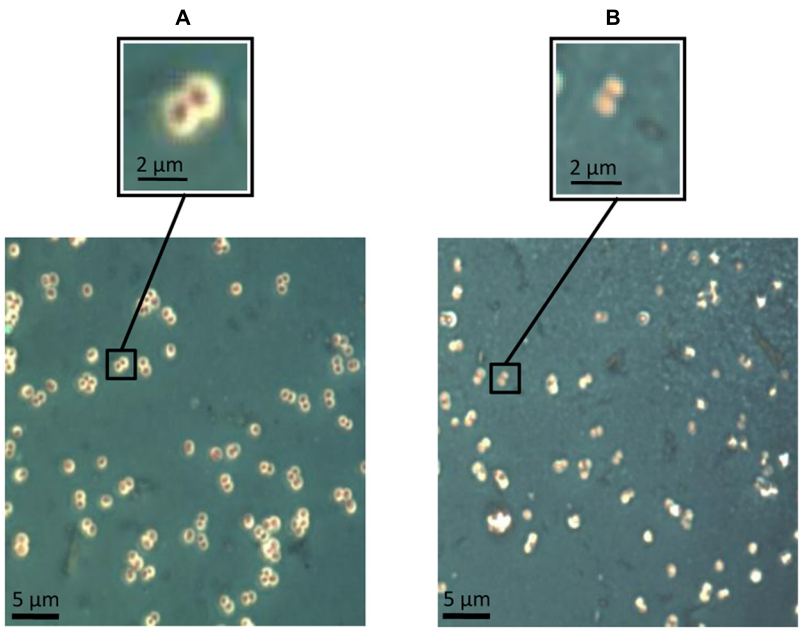

FIGURE 4 | Maneval's staining of extracellular material in exponential-phase cultures of *S. epidermidis* F12 (A) before treatment and (B) after treatment with 0.15 μM of Dpo7 for 3 h at 37°C. A magnification of the figure allows for the comparison of the presence/absence of polysaccharide matrix represented by a white halo surrounding the cell.

increasing temperature (25, 30, and 44% of biomass was removed at 22, 32, and 37°C, respectively). Dpo7 was found to be quite thermostable as heat treatments up to temperatures of 70°C did not significantly affect the enzymatic activity (**Figure 3A**). Dpo7 was also stable in a range of pH from 6 to 8 (**Figure 3B**). After 90 days of storage a 4°C, no decrease of activity was observed (data not shown).

A qualitative approach about the potential of Dpo7 to degrade the extracellular material formed by *S. epidermidis* F12 in liquid cultures was obtained by staining and optical microscopy (**Figure 4**). Cells grown to exponential phase were incubated with 0.15 μM of Dpo7 for 3 h at 37°C. Microscopic analysis showed that non-treated cells are closely associated into small clusters surrounded by a thin layer of negatively stained capsular material (**Figure 4A**). After treatment with exopolysaccharide (EPS) depolymerase Dpo7, the capsule is generally thinner or drastically disrupted, and remaining capsular material devoid of bacteria can be observed (**Figure 4B**).

The Exopolysaccharide is the Main Substrate of Dpo7

To determine the specificity of Dpo7 for the components of *S. epidermidis* biofilms, we tested its activity against biofilms with different biochemical matrix compositions. Our *S. epidermidis* and *S. aureus* strains collection was tested for matrix composition by using treatment with either $NaIO_4$, proteinase K and DNaseI,

TABLE 2 | Estimation of the extracellular components in biofilms formed by staphylococcal strains through the percentage of biofilm removing after treatment.

	Strain	% of removed biofilm		
		$NaIO_4$	Proteinase K	DNase I
S. epidermidis	F12	77.7 ± 9.0	1.9 ± 0.3	14 ± 5.9
	B*	76.2 ± 8.2	2.7 ± 1.4	14.9 ± 3.1
	DH3LIK	85 ± 3.8	5.9 ± 1.9	6.2 ± 1.2
	YLIC13	71.8 ± 1.3	8 ± 1.8	22 ± 2.4
	Z2LDC14	73.4 ± 2.8	7.7 ± 2.7	32.3 ± 5.5
	DG2n*	75.2 ± 4.6	3.9 ± 1.5	24.7 ± 10.4
	ASLD1	73.3 ± 5.6	0.5 ± 0.3	24.2 ± 7.1
	LO5081	81.4 ± 9.6	9.7 ± 3.3	36.6 ± 3.3
	LV5RB3	76.7 ± 4.2	8.1 ± 7.7	23.1 ± 6.4
	LO5RB1	86.2 ± 6.0	6.1 ± 2.7	23.1 ± 9.5
S. aureus	15981*	88.7 ± 2.5	3.5 ± 0.9	1.6 ± 0.8
	V329*	3.2 ± 5.1	88.7 ± 0.1	49.4 ± 2.2

Results published in Gutiérrez et al. (2014).

specific for exopolysaccharidic, proteinaceous and DNA matrix, respectively. An estimation of the matrix composition was made as function of the percentage of biofilm removal after treatments. The results showed that all *S. epidermidis* strains tested produced a matrix composed mainly of EPS as the highest percentage of biofilm removal was obtained after treatment with $NaIO_4$. Strains *S. aureus* 15981 and *S. aureus* V329 formed EPS and proteinaceous biofilms, respectively (**Table 2**). Dpo7 assays against 24 h-biofilms of *S. epidermidis* strains showed a significant reduction in adhered cells from biofilms containing an EPS matrix (**Figure 5A**). The decrease ranged from 1 log-unit in the biofilm formed by *S. epidermidis* LO5RB1 to 2.3 log-units in *S. epidermidis* DH3LIK biofilm. Moreover, an increase in the number of cells was observed in the supernatant (data not shown). As expected, the number of adhered cells in *S. aureus* V329 biofilms was not reduced due to its proteinaceous biofilm matrix but a clear reduction was observed in *S. aureus* 15981 (**Figure 5A**). These results were confirmed by staining the total biomass with crystal violet. Dpo7 activity against EPS biofilms showed a reduction of biomass ranging from 31% in *S. epidermidis* ASLD1 to 75% in *S. epidermidis* LO5081, remaining unaffected in *S. aureus* V329 (**Figure 5B**).

Dpo7 Prevents the Biofilm Formation on Polystyrene Surfaces

In addition to the evaluation of Dpo7 to prevent biofilm formation in staphylococcal strains, we tested the effect of the protein on the planktonic growth of the strains. Microwell plates filled with TSBg supplemented with Dpo7 (0–1.5 μM) were inoculated with staphylococcal strains and the growth at 37°C was monitored by DO_{600} after 24 h of incubation. No statistically significant differences were observed between planktonic cultures treated and non-treated with Dpo7 at any assayed concentration. **Figure 6A** shows results obtained at 0.15 μM Dpo 7 but higher concentrations (up to 1.5 μM) did not affect planktonic growth.

In order to assess the ability of Dpo7 to prevent biofilm formation, staphylococcal strains were also grown in the presence of different concentrations (0–1.5 μM) of Dpo7 and checked for biofilm formation by crystal violet staining. The presence of 0.15 μM of Dpo7 inhibited biofilm formation as total biomass adhered to the polystyrene surface decreased from 27% in *S. epidermidis* LV5RB3 to 85% in *S. epidermidis* Z2LDC14

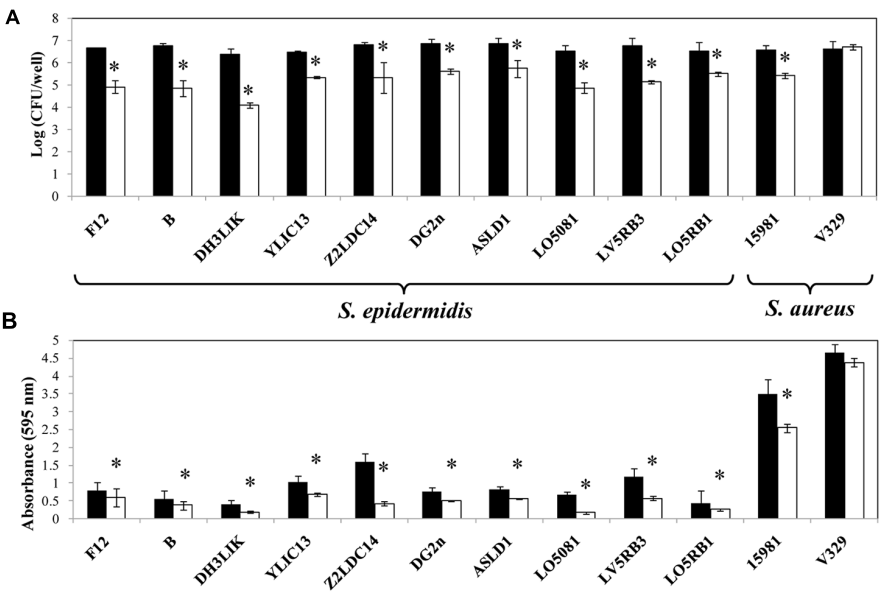

FIGURE 5 | Removal of 24 h *S. epidermidis* and *S. aureus* biofilms after addition of Dpo7 (0.15 μM) for 3 h at 37°C. (A) Adhered cultivable bacteria and **(B)** crystal violet staining of control biofilms (black) and treated biofilms (white). Means and standard deviations were calculated from three biological replicates. Bars having an asterisk are significantly different from the control (ANOVA; $P < 0.05$).

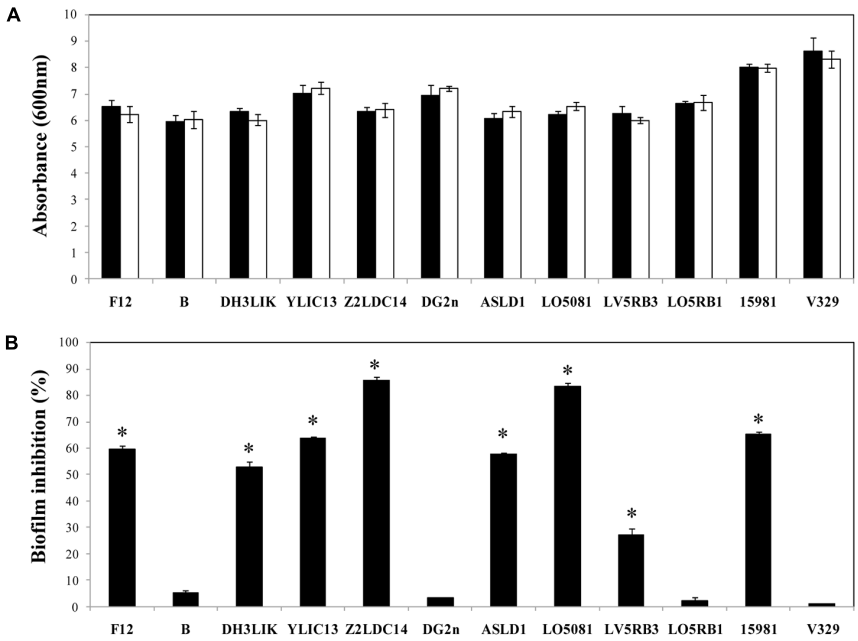

FIGURE 6 | Biofilm growth inhibition in presence of Dpo7 (0.15 μM). (A) Planktonic growth of the cultures was determined by absorbance measurement at 600 nm after 24 h of incubation at 37°C. **(B)** Percentage of biofilm inhibition was calculated by crystal violet staining of adhered cells after growth of 24 h biofilms. Black bars represent the control values and white bars represent the values of wells treated with Dpo7. Means and standard deviations were calculated from three biological replicates. Bars having an asterisk are significantly different from the control (ANOVA; $P < 0.05$).

(**Figure 6B**). Higher concentrations of Dpo7 (up to 1.5 μM) did not improve these results (data not shown). However, the presence of the Dpo7 did not affect biofilm formation in the case of *S. epidermidis* B, DG2n, LO5RB1 and *S. aureus* V329 (**Figure 6B**).

DISCUSSION

In this study, we have purified a phage-derived EPS depolymerase, Dpo7, which degrades the EPS matrix of *S. epidermidis* and *S. aureus* biofilms. Apart from Dispersin B, an *N*-acetylglucosaminidase enzyme produced by *Aggregatibacter actinomycetemcomitans* (Izano et al., 2008), no other enzymes degrading staphylococcal biofilm matrix formed by polysaccharides have been described so far. This finding prompted us to study Dpo7 as a strategy for controlling the colonization and biofilm formation by staphylococcal pathogens. The presence of depolymerases is common among phages, playing a putative role in phage adsorption and infection (Cornelissen et al., 2011, 2012). Similar proteins to Dpo7 are also present in three other *Siphoviridae* phages infecting *S. epidermidis* (Daniel et al., 2007; Gutiérrez et al., 2012). All phage EPS depolymerases characterized to date are enzymes that hydrolyze polysaccharides or polysaccharide derivatives and are common constituents of the tail spikes (Yan et al., 2014). For instance, phage EPS depolymerases include endorhamnosidases (Muller et al., 2008; Andres et al., 2010), alginate lyases (Glonti et al., 2010), endosialidases (Jakobsson et al., 2007) and hyaluronidases (Smith et al., 2005). Regarding Dpo7, it is reasonable to hypothesize that it is associated to the capsid. Indeed, Dpo7 amino acid sequence showed 99% homology with a pre-neck appendage protein encoded by *S. epidermidis* bacteriophage CNPH82 and high similarity in the tridimensional structure with phi29 gp12 neck protein (Gutiérrez et al., 2012).

The protein Dpo7 contains a predicted pectin lyase domain, the structure of which is composed of a right-handed β-helix (Gutiérrez et al., 2012). Proteins containing these repeats are usually enzymes with polysaccharide substrates, and this topology is shared by several proteins, including bacterial pectate lyases, fungal and bacterial galacturonases. Also, the phage tail spike protein HylP1, encoded by l-like phage infecting *Streptococcus pyogenes,* is a hyaluronidase with a similar structure (Smith et al., 2005). Our results show that Dpo7 is involved in the degradation of the EPS biofilm matrix of staphylococcal strains. The ability of Dpo7 to disperse polysaccharide staphylococcal preformed biofilms, but not the proteinaceous biofilm formed by the strain *S. aureus* V329 supports this hypothesis. Degradation of proteins associated with *S. aureus* biofilm formation and host–pathogen interaction was previously described by *S. epidermidis* protease Esp (Sugimoto et al., 2013).

In spite of the low identity (13%) and similarity (21%) percentages between the amino acid sequences of Dpo7 and DispersinB®, the enzymatic activity and the optimum conditions of Dpo7 were similar to those of DispersinB®. Of note, the last one has been proved to be effective removing over 85% of the biomass of some staphylococcal biofilms when applying 40 or 50 $\mu g\ ml^{-1}$ (Kaplan et al., 2003; Izano et al., 2008; Turk

et al., 2013). The activity of Dpo7 was not dependent on the ability of the strains to form weaker or stronger biofilms but on the nature of the matrix. Indeed, the highest percentage of biofilm removal was obtained against *S. epidermidis* Z2LDC14 and *S. epidermidis* LO5081, which showed a high and a moderate ability to form biofilm, respectively, from among *S. epidermidis* strains. We also observed that concentrations over 0.15 μM did not improve biofilm dispersion even if time of treatment was increased. The maintenance of the biofilm after the treatment could be due to the heterogeneity of the biofilm matrix or to a low diffusion of the protein within the biofilm. In EPS biofilms, although the majority of the extracellular matrix is composed of polysaccharides, interactions with eDNA and proteins should not be discarded (Izano et al., 2008) therefore limiting the potential of Dpo7 to remove the biofilm completely. This limitation was also observed when using DispersinB® to remove biofilms formed by *S. pseudintermedius* (Turk et al., 2013). The activity of this enzyme was dependent on the proportion of the constituents of the biofilm matrix, which varies with the growth conditions and among the different strains (Chaignon et al., 2007). Moreover, PIA/PNAG molecules do not have a definite structure and are not well-defined substrates, varying in length and charge (Mack et al., 1996). It has been demonstrated that the chain length of PIA/PNAG increased the catalytic efficiency of DispersinB® (Fazekas et al., 2012). Similarly, Dpo7 may control EPS staphylococcal biofilms, but its activity could be limited by the nature of the biofilm matrix, requiring the use of combined treatments to completely eradicate the biofilm. Combined treatments of DispersinB® with antibacterials such as triclosan or cefamandole nafate reduced colonization and biofilm formation by *S. aureus*, *S. epidermidis*, and *E. coli* in coated medical devices (Donelli et al., 2007; Darouiche et al., 2009). Moreover, the development of an engineered enzymatic bacteriophage (T7) modified to express DispersinB® during infection, enhanced the ability of the phage to reduce biofilms formed by *E. coli* (Lu and Collins, 2007).

The relatively low activity of Dpo7 against extracellular material allows us to speculate about the role of this protein in the viral particle. Dpo7 might be implicated in the access of phage to the bacterial surface without a total disaggregation of the biofilm structure. Moreover, cells susceptible to phage attack should be located in the outer zone of biofilms, where nutrients and oxygen availability allow them to grow actively. Therefore, the biological function of Dpo7 might not be the highly effective degradation of extracellular EPS matrix but to open a local trajectory to enable virion/cell contact.

A major finding of this study was that treatment of polystyrene surfaces with Dpo7 inhibits the colonization by staphylococcal strains. It seems that removal of the extracellular material at the early stages of the culture is sufficient to prevent the adhesion of most strains to the polystyrene surfaces. As deduced from our results, Dpo7 is also able to remove extracellular material from planktonic cells, resulting in the inhibition of biofilm formation. This is in accordance with previous results obtained adding DispersinB® for biofilm prevention of several bacteria specific strains such as *S. epidermidis, E. coli,* or *Yersinia pestis,* which produce polysaccharidic matrixes (Itoh et al., 2005), and it was also useful to reduce but not completely inhibit biofilm formation of several strains of *S. pseudintermedius* (Turk et al., 2013).

All these results in addition to the lack of an antibacterial effect of Dpo7 indicates that this enzyme would be used to remove the extracellular material in staphylococcal strains to reduce their virulence, since it has been shown that *S. epidermidis* mutants deficient in the ability to synthesize PIA/PNAG are avirulent in animal models of infection (Shiro et al., 1994). Similarly, biofilm production by *S. aureus* is important for high-level virulence in murine models of systemic infection, likely due to protection of cells from host defenses (Kropec et al., 2005). Thus, Dpo7 may also be considered as an antivirulence compound. Generally, resistance development against antivirulence compounds is expected to be lower than against antibacterial compounds, as the bacteria are only disarmed and not killed (Allen et al., 2014). The efficacy of anti-biofilm enzymes has been proven with DispersinB® for the treatment of infectious diseases in a chronic wound mouse model of MRSA infection (Gawande et al., 2014), and in the reduction of colonization of catheters by *S. aureus* (Darouiche et al., 2009). The removal of capsular polysaccharide by the phage-encoded enzyme endosialidase E reduces the virulence of *E. coli* K1 in a non-invasive model in neonatal rats (Mushtaq et al., 2005). Similarly, depolymerase enzymes encoded by phages infecting *Erwinia amylovora* have been proposed in the control of plant diseases (Kim et al., 2004). In addition, the removal of polymeric extracellular material might improve the action of antibiotics against bacteria forming biofilms, since physical interference could be avoided (Farber et al., 1990; Alkawash et al., 2006).

This study supports that phages possess structural components such as the novel EPS depolymerase (Dpo7) that inhibit biofilm formation and disperses preformed biofilms of *S. epidermidis* and *S. aureus*. These kinds of proteins might constitute a new approach to remove biofilms, thus improving the treatment of biofilm-associated recalcitrant infections.

AUTHOR CONTRIBUTIONS

DG, YB, LR-R, BM, AR, RL, and PG conceived and designed the experiments. DG performed the experiments. DG, YB, AR, PG, and RL analyzed the data. DG, YB, LR, BM, AR, RL, and PG wrote the paper.

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PL3 Amidase, a Tailor-made Lysin Constructed by Domain Shuffling with Potent Killing Activity against Pneumococci and Related Species

Blas Blázquez[1†], Alba Fresco-Taboada[1†], Manuel Iglesias-Bexiga[2,3], Margarita Menéndez[2,3] and Pedro García[1,3]*

[1] Departamento de Microbiología Molecular y Biología de las Infecciones, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain, [2] Departamento de Química-Física Biológica, Instituto Química-Física Rocasolano, Consejo Superior de Investigaciones Científicas, Madrid, Spain, [3] CIBER de Enfermedades Respiratorias, Madrid, Spain

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University of Limoges, France

***Correspondence:**
Pedro García
pgarcia@cib.csic.es

[†] These authors have contributed equally to this work.

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The emergence and spread of antibiotic-resistant bacteria is pushing the need of alternative treatments. In this context, phage therapy is already a reality to successfully fight certain multiresistant bacteria. Among different phage gene products, murein hydrolases responsible of phage progeny liberation (also called lysins or endolysins) are weapons that target specific peptidoglycan bonds, leading to lysis and death of susceptible bacteria when added from the outside. In the pneumococcal system, all but one phage murein hydrolases reported to date share a choline-binding domain that recognizes cell walls containing choline residues in the (lipo)teichoic acids. Some purified pneumococcal or phage murein hydrolases, as well as several chimeric proteins combining natural catalytic and cell wall-binding domains (CBDs) have been used as effective antimicrobials. In this work we have constructed a novel chimeric *N*-acetylmuramoyl-L-alanine amidase (PL3) by fusing the catalytic domain of the Pal amidase (a phage-coded endolysin) to the CBD of the LytA amidase, the major pneumococcal autolysin. The physicochemical properties of PL3 and the bacteriolytic effect against several pneumococci (including 48 multiresistant representative strain) and related species, like *Streptococcus pseudopneumoniae*, *Streptococcus mitis*, and *Streptococcus oralis*, have been studied. Results have shown that low doses of PL3, in the range of 0.5–5 μg/ml, are enough to practically sterilize all choline-containing strains tested. Moreover, a single 20-μg dose of PL3 fully protected zebrafish embryos from infection by *S. pneumoniae* D39 strain. Importantly, PL3 keeps 95% enzymatic activity after 4 weeks at 37°C and can be lyophilized without losing activity, demonstrating a remarkable robustness. Such stability, together with a prominent efficacy against a narrow spectrum of human pathogens, confers to PL3 the characteristic to be an effective therapeutic. In addition, our results demonstrate that the structure/function-based domain shuffling approach is a successful method to construct tailor-made endolysins with higher bactericidal activities than their parental enzymes.

Keywords: lysin, pneumococcus, phage therapy, chimeric protein, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus pseudopneumoniae*

INTRODUCTION

Discovery of penicillin and other antibiotics allowed effective treatment of infectious diseases, which provoked a tremendous impact on public health. However, it has been learned that sooner or later bacteria are capable of acquiring resistance to practically every known antibiotic. This resistance is readily transferred to other bacteria and, at the end, there is a continuous warfare between the ability of bacteria to resist any new antimicrobial and the armamentarium of new weapons to overcome treatment failures and kill the targeted bacteria. In this context, the use and abuse of antibiotics in the last years have led to a substantial rise of bacterial multiresistance and this worrying situation runs parallel with the scarcity of new antimicrobials in the pharmaceutical pipeline (Spellberg et al., 2015).

Among the main human pathogens, *Streptococcus pneumoniae* continues to be a major cause of morbidity and mortality worldwide —causing more deaths than any other infectious disease —being children younger than 5 years old, the elderly, and immunocompromised people the major groups at risk. Pneumococcal diseases range from mild infections, such as otitis media and sinusitis, to more severe diseases such as pneumonia (either invasive or not), septicemia, and meningitis. Despite the availability of vaccines and antibiotics, a recent report estimated that pneumococcus is still responsible for approximately 1.3 million deaths annually (Walker et al., 2013). For decades, the standard treatment of pneumococcal infections has been penicillin, to which this species was exquisitely sensitive. However, a widespread increase of pneumococci resistant to most antibiotics (except to vancomycin) has been progressively observed (Kim et al., 2016). The tendency on the emergence of multidrug resistance pathogens is an increasingly global economic and healthcare crisis, and this situation is pushing to find alternative approaches for combating such pathogens, *S. pneumoniae* being one of the more clear examples (Huttner et al., 2013).

Bacteriophage-encoded lytic enzymes (or endolysins) are murein hydrolases that selectively break different bonds of peptidoglycan, thereby enabling the release of progeny virions at the end of the infection cycle of the majority of double-stranded DNA bacteriophages. Purified endolysins, and bacterial autolysins as well, can be applied exogenously to brake the bacterial cell wall in an effective and selective way. This novel class of antibacterials, also known as enzybiotics, presents important advantages over classical antibiotics, e.g., narrow spectrum of susceptible bacteria, rapid killing of stationary and logarithmically growing bacteria, and low probability to bacterial resistance (Pastagia et al., 2013). In addition, lysins can also eliminate bacteria from mucous membranes and bacterial biofilms, which are major reservoirs and routes of infection (Pastagia et al., 2011; Díez-Martínez et al., 2015). Typically, lysins from Gram-positive bacteria and their bacteriophages consist of a two-domain structure, but some of them have multiple hydrolytic domains or distinct types of cell wall-binding domains (CBDs; Rigden et al., 2003; Nelson et al., 2012). The stringent range of activity is primarily linked to the specificity of binding of the CBDs (Hermoso et al., 2007; Gilmer et al., 2013). However, the net charge of the domains and the fine architecture of the bacterial envelope contribute as well (Low et al., 2011; Díez-Martínez et al., 2013). Besides, the intrinsic activity of the catalytic domain, the strength of attachment to the cell wall, and the overall protein structure determine the actual lysis rate.

In the last years, several reports of endolysins showing strong lethal activity against relevant Gram-positive pathogens have been published, including *Staphylococcus aureus* (Rashel et al., 2007; Gilmer et al., 2013), enterococci (Yoong et al., 2004), the spore formers *Bacillus* and *Clostridium* genera (Nakonieczna et al., 2015), and even some Gram-negative pathogens like *Acinetobacter baumannii* (Lood et al., 2015). Specifically in pneumococcus, endolysins like the Cpl-1 and Cpl-7 lysozymes and derived chimeras (Díez-Martínez et al., 2013, 2015), or the Pal *N*-acetylmuramoyl-L-alanine amidase (NAM-amidase; EC 3.5.1.28; Loeffler et al., 2001; Jado et al., 2003) have been proved to kill efficiently several strains *in vitro* and *in vivo*. Moreover, the bactericidal effect of the major pneumococcal autolysin, the LytA NAM-amidase, against encapsulated *S. pneumoniae* cells has also been demonstrated (Rodríguez-Cerrato et al., 2007; Díez-Martínez et al., 2013). Pal and LytA have unrelated catalytic domains belonging to *Amidase_5* and *Amidase_2* families, respectively, which are fused to homologous choline-binding domains (66% sequence identity) that anchor to the phosphocholine residues of pneumococcal (lipo)teichoic acids (Sheehan et al., 1997). Both choline-binding domains are made up of six sequence-conserved repeats and a C-terminal tail, where choline moieties bind at the interface of every two-consecutive repeats, as deduced by the elucidation of the crystallographic structures of full-length LytA and its isolated choline-binding domain (Fernández-Tornero et al., 2001; Li et al., 2015). Besides, LytA contains a non-canonical choline-binding site in the first repeat of the choline-binding domain (Mellroth et al., 2014; Li et al., 2015). Based on the previous structural and functional knowledge of both NAM-amidases (Varea et al., 2000 and references therein; Fernández-Tornero et al., 2001; Varea et al., 2004; Li et al., 2015), we have constructed a novel chimeric lysin, PL3, by shuffling the catalytic domain of Pal with the choline-binding domain of LytA. PL3 turned out to be a potent enzybiotic against pneumococci and other choline-containing Gram-positive pathogens, and its lethality against pneumococcal encapsulated and multiresistant isolates was higher than those of the parental enzymes. In addition, *in vitro* bactericidal activity of PL3 has also been confirmed *in vivo* using a zebrafish embryo infection model.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in **Table 1**. Pneumococcal cultures were grown at 37°C without aeration in C medium supplemented with 0.08% (w/v) yeast extract (C + Y medium; Lacks and Hotchkiss, 1960). Other Gram-positive bacteria were grown in brain heart infusion broth (Becton, Dickinson and Company) at 37°C without shaking. *Escherichia coli* strains were grown in LB medium with aeration

TABLE 1 | Bacterial strains and plasmids.

Strains or plasmids	Genotype or description[a]	Reference[b]
Strains		
Escherichia coli BL21(DE3)	F$^-$, *ompT, hsdSB* (r_B^- m_B^-), *gal, dcm*, λDE3 (harboring gene *1* of the RNA polymerase from the phage T7 under the *PlacUV5* promoter)	Sambrook and Russell, 2001
Streptococcus pneumoniae		
R6	Standard laboratory strain, non-encapsulated	Hoskins et al., 2001
P046	R6 but *lytA, lytC*	Moscoso et al., 2006
D39	Serotype 2	Lanie et al., 2007
P007	R6 derivative, serotype 3	Domenech et al., 2009
P008	R6 derivative, serotype 4	Moscoso et al., 2006
48	Serotype 23F; penicillin MIC = 16 mg/ml; erythromycin MIC > 128 mg/ml; ciprofloxacin MIC = 1 mg/ml; levofloxacin MIC = 1 mg/ml; chloramphenicol MIC = 4 mg/ml; tetracycline MIC > 64 mg/ml	Soriano et al., 2008
Streptococcus mitis^T	Type strain	NCTC 12261
Streptococcus mitis SK598	Biovar 1 strain with ethanolamine-containing C-polysaccharide	Bergström et al., 2003
Streptococcus oralis^T	Type strain	NCTC 11427
Streptococcus pseudopneumoniae^T	Type strain	ATCC BAA-960
Plasmids		
pET29a(+)	Expression vector; Km^R	Novagen
pET29-PL3	pET29a(+), *pl3*; Km^R	This study
pMSP11	Recombinant plasmid with *pal*	Sheehan et al., 1997
pMMN1	Recombinant plasmid with *lytA*	Moscoso et al., 2011

[a]*MIC, minimal inhibitory concentration.*
[b]*NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection.*

at 37°C, supplemented with kanamycin (Km; 50 μg/ml) when required.

Cloning, Expression, and Purification of PL3

The Pal encoding region was PCR amplified with primers 5'Nde_Pal (GGAGGGAAGACATATGGGAGTCGATATTGAAAAAGG, where the NdeI site is underlined) and 3'GYM_Pal (CGGTCTGCAAGCATGTAGCCTTGGTCGTCAAAG), using pMSP11 as template (Sheehan et al., 1997). The LytA encoding region was PCR amplified with primers 5'LA_LytA (CTTTGACGACCAAGGCTACATGCTTGCAGACCG) and 3'BamHI_LytA (CGCGGATCCTTATTTTACTGTAATCAAGCCATCTG, where the BamHI site is underlined) using pMMN1 as template (Moscoso et al., 2011). The resulting PCR products were used for a third PCR round to amplify the chimeric PL3 encoding gene, which was digested with NdeI and BamHI and cloned into pET29a(+) previously treated with the same enzymes. The resulting recombinant plasmid, pET29-PL3, was sequenced to ensure the accuracy of the insert and transformed into *E. coli* BL21(DE3). For overproduction of PL3, transformed cells were incubated to an optical density at 600 nm (OD_{600}) of 0.6. Isopropyl-β-D-galactopyranoside (0.1 mM) was then added, and incubation continued for 4 h at 37°C. Cells were harvested by centrifugation (10000 × *g*, 5 min), resuspended in 20 mM sodium phosphate buffer (hereafter, PB), 0.5 M NaCl, pH 6.9, disrupted in a French pressure cell and ultracentrifuged (50000 × *g*, 45 min) to remove cell debris. Streptomycin sulfate (Sigma; 2%, w/v) was added to the protein extract and the mixture was incubated for 15 min at 4°C with slow stirring to facilitate DNA precipitation. The insoluble fraction was removed by ultracentrifugation (50000 × *g*, 45 min) at 4°C, and PL3 was purified from the supernatant by affinity chromatography using DEAE-cellulose (Sanz and García, 1990) followed by size exclusion chromatography on dextran-agarose (HiLoad 16/60 Superdex 200 PG column, GE Healthcare) to remove large protein aggregates. Briefly, PL3 fractions eluted from the affinity column were pooled, dialyzed against PB, pH 6.8, and subjected to gel filtration using the same buffer at a flow rate of 0.8 ml/min. The purity and state of the PL3 samples were checked by 12% SDS-PAGE and mass spectrometry (MALDI-TOF). Large-aggregate free fractions of PL3 were pooled, dialyzed against PB containing 100 mM NaCl, 25 mM choline, pH 6.8, and stored at −20°C. Before use, the protein was dialyzed against PB, pH 6.8, supplemented with 1 mM β-mercaptoethanol or 10 mM 1,4-dithiothreitol (DTT) when required. PL3 concentration was determined spectrophotometrically using the theoretical molar absorption coefficient at 280 nm (133855 M^{-1} cm^{-1}, considering cysteine residues in the oxidized state).

Mass Spectrometry

Purified PL3 samples were analyzed by MALDI-TOF in a Voyager DEPRO (Applied Biosystems), as described elsewhere (Moreno et al., 2008). A grid voltage of 89%, a 0.25 ion guide wire voltage, and a delay time of 400 ns in the linear positive-ion mode were used. External calibration was performed with carbonic anhydrase (29024 Da) and enolase (46672 Da) from Sigma, covering an *m/z* range of 10000–80000 units.

Circular Dichroism (CD)

Circular dichroism spectra were recorded at 20°C with a J-810 spectropolarimeter (Jasco Corporation) equipped with a Peltier-type cell holder, using 1-mm (far-UV) or 10-mm (near-UV) path-length quartz cells and protein concentrations of 0.13 and 0.44 mg/ml, respectively (Bustamante et al., 2010). The buffer contribution was subtracted from the raw data and the corrected spectra were converted to mean residue ellipticities (Θ) using an average molecular mass per residue of 104.5. Spectra acquisition and analysis were carried out with the Spectra Manager software.

PL3 titration with choline was performed by measuring the CD spectra at varying choline concentrations and plotting the ellipticity variation at selected wavelengths as a function

of choline concentration. To minimize errors, titrations were carried out by serial addition of small volumes of concentrated choline stocks to the same protein sample (less than 10% total volume increase). Choline stock concentrations were measured by differential refractometry (Usobiaga et al., 1996).

Analytical Ultracentrifugation

Sedimentation velocity experiments were carried out in an Optima XL-A analytical ultracentrifuge (Beckman Coulter) at 20°C. Measurements were performed in PB, pH 6.8, at 45000 rpm using cells with double sector Epon-charcoal centerpieces (0.11 mg/ml PL3). Differential sedimentation coefficients were calculated by least-squares boundary modeling of the experimental data, and normalized to values in water at 20°C ($s_{20,w}$), with the program SEDFIT (Brown and Schuck, 2006). The fractional friction coefficients (f/f_0) and the Stokes radii (R_s), related to the protein hydrodynamic shape, were calculated from the molecular masses and $s_{20,w}$ values using the partial specific volumes and hydration coefficients estimated from the amino acid sequence with the SEDNTERP program (Laue et al., 1992).

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry measurements were performed at a heating rate of 60°C/h in a VP-DSC microcalorimeter (Microcal, Inc.), under an extra constant pressure of 1.8 atm, at 0.4 mg/ml PL3. Origin DSC software (Microcal) was used for data acquisition and analysis. The excess heat capacity function was obtained after subtraction of the buffer-buffer base line registered before each protein scan. Reheating of previously scanned samples showed that thermal denaturation of PL3 was totally or partially irreversible, depending on the buffer pH.

In Vitro Cell Wall Activity Assay

Purified PL3 was checked for *in vitro* cell wall degradation using [*methyl*-^{3}H] choline-labeled pneumococcal cell walls as substrate, following a previously described method (Mosser and Tomasz, 1970). Briefly, 10 μl of enzyme at the appropriate dilution was added to the reaction sample containing 240 μl of PB, 100 mM NaCl, 10 mM DTT, pH 6.8, and 10 μl of radioactively labeled cell walls (≈15000 cpm). After 15 min incubation at 37°C the reaction was stopped by adding 10 μl formaldehyde (37%, v/v) and 10 μl bovine serum albumin (4%, w/v). The pellet was removed by centrifugation (12000 × *g*, 15 min), and the enzymatic activity was quantified by measuring the radioactivity in 200 μl of the supernatant with a liquid scintillation counter (LKB Wallac). One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzes the hydrolysis (solubilization) of 1 μg of cell wall material in 15 min (Höltje and Tomasz, 1976). Activity assays at different pHs were performed in 20 mM sodium phosphate (pH 5.7–8.0) or 20 mM HCl-Tris buffers (pH 8.0–9.0).

Bactericidal Assay

Log-phase bacteria were grown to an $OD_{550} \approx 0.3$, cooled on ice for 5 min, centrifuged, washed with PB, 100 mM NaCl, pH 6.8, and adjusted to an $OD_{550} \approx 0.6$ (10^8–10^9 colony forming units (CFUs) per ml) in the same buffer supplemented with 10 mM DTT. Afterward, resuspended bacteria were transferred into plastic tubes and PL3 was added. Controls were always run in parallel, replacing the added enzyme with buffer. Samples were incubated at 37°C for 1 h, the turbidity decrease (OD_{550}) was measured, and viable cells were determined using blood agar plates at the end of incubation. For each sample, a 10-fold dilution series was prepared, 10 μl of each dilution was plated, and colonies were counted after overnight incubation at 37°C. Only dilutions with 30–300 colonies were considered, and in assays where the bactericidal effect was high, 100 μl of undiluted suspensions were plated and colonies counted.

Activity of PL3 in Different Phases of the Growth Curve

Streptococcus pneumoniae R6 and P046 strains were incubated until exponential phase of growth and diluted to an OD_{550} of 0.06. Then, cultures were divided in aliquots and PL3 (2.7 μg/ml, final concentration) was added at early exponential phase ($OD_{550} \approx 0.15$), late exponential phase ($OD_{550} \approx 0.4$), or stationary phase of growth ($OD_{550} \approx 0.7$), with or without 10 mM DTT in the medium. Viable cells of treated and untreated samples were counted at 210 min after culture initiation, as explained above.

Zebrafish Embryo Infection Assay

This study was conducted at The Zebrafish Lab[1], using wild-type zebrafish embryos that were maintained according to standard protocols (Westerfield, 2007). Briefly, zebrafish embryos were dechorionated at 24 h post fecundation by treatment with pronase (2 mg/ml) for 2 min. At 48 h post fecundation, embryos were individually distributed in 96-well plates and incubated in 50 μl of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$, pH 7) at 28°C in the presence of either alive or heat-killed D39 pneumococcal cells (≈10^8 CFU/ml) for 8 h. The effect of adding 20 μg of PL3 or 1 mM DTT to uninfected embryos was also tested. Afterward, infected embryos were extensively washed with E3 medium, to remove bacteria, transferred, together with the controls, to new 96-well microtiter plates containing autoclaved E3 fresh medium supplemented with different amounts of PL3 and 1 mM DTT — or the same volume of buffer (controls) — before continuing incubation at 28°C under sterile conditions. Mortality was followed in all samples for 5 days, adding fresh E3 medium without DTT every day. Zebrafish embryos were considered dead when no movement was observed, even if a heartbeat was observed. Opacification of the larvae was always found to follow shortly. Bacterial infection was previously ascertained as the real cause of embryo death by locating fluorescent bacterial signals around the gills (Díez-Martínez et al., 2013).

Statistical Analysis

All data are representative of results obtained from repeated independent experiments, and each value represents the

[1]http://www.thezebrafishlab.com

mean ± standard deviations for three replicates. In the case of the zebrafish embryo assay, the results from four independent experiments were combined to evaluate a total of 256 embryos for controls and for each lysin-treated group. Statistical analysis was performed by using two-tailed Student's *t*-test (for two groups), whereas analysis of variance (ANOVA) was chosen for multiple comparisons. GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

RESULTS

Design and Production of the PL3 Chimera

The goal of this work was to construct a new chimeric lysin specifically directed against *S. pneumoniae* and other choline-containing Gram-positive bacteria, with higher activity and stability than the parental enzymes. In this context, we have recently shown how the substitution of the Cpl-7 CBD by a different domain with higher affinity for the substrate resulted in an extremely powerful lysin against pneumococci, i.e., the Cpl-711 chimera (Díez-Martínez et al., 2015). With this aim, we thoroughly analyzed the structural, enzymatic, and bactericidal properties of natural and chimeric lysins from the pneumococcal system, which includes the bacterium and its phages. Thus, we decided to construct the PL3 chimera by combining the catalytic *Amidase_5* domain (PF05382) from Pal, encoded by the bacteriophage Dp-1, and the C-terminal region of the CBD from the major pneumococcal autolysin LytA, which is a member of the *Amidase_2* family (PF01510). The approach was based on the following rationale: (i) both lysins are effective antimicrobials against pneumococci (Jado et al., 2003; Rodríguez-Cerrato et al., 2007); (ii) the catalytic module of Pal is less negatively charged than that of LytA, which might facilitate the lysis from the outside (Low et al., 2011); (iii) saturation of choline-binding sites and choline-induced dimerization, key for lytic activity, occur in LytA at lower ligand concentration than in Pal (Medrano et al., 1996; Varea et al., 2000, 2004); (iv) preservation of Pal overall modular structure in the chimera could be achieved by conserving the linker and the two first choline-binding repeats of Pal (61% sequence identity to those of LytA); and (v) the new chimera will combine the most structurally stable domains of the parental enzymes: the CBD from LytA and the catalytic domain of Pal (Varea et al., 2000, 2004). A comparative scheme of PL3 and the parental Pal and LytA enzymes is shown in **Figure 1** whereas domain and linker charges are given in Supplementary Figure S1 together with amino acid sequence.

Cloning, overproduction and purification of PL3 were carried out as detailed in the Section "Materials and Methods," with a yield of ≈110 mg per liter of culture. The protein eluted from the DEAE-cellulose column showed two close bands (R and O) when analyzed under non-reducing conditions by SDS-PAGE. Nevertheless, the faster migrating band (O) disappeared upon pretreatment of the sample with 10 mM DTT (or by adding 1 mM β-mercaptoethanol) to the sample-loading buffer (**Figure 2A**), and the remaining band (R) corresponded with the expected mobility from the theoretical molecular mass of PL3 (34287 Da) and the experimental value measured by MALDI-TOFF mass (34151.3 Da; Met1 is processed). This observation, indicative of an intra-molecular disulphide bridge between the two cysteine residues (Cys34 and Cys112) of PL3 (and Pal) catalytic domain, was consistent with the stimulation of Pal (García et al., 1983) and PL3 (see below) activities by reducing agents. In addition, size-exclusion chromatography of PL3 samples revealed the presence of different association states (**Figure 2B**), including large protein aggregates eluting at the void volume of the column (peak 1), and a likely oxidized form of PL3 (peak 5) observed only when PB used for the protein preequilibration did not contain β-mercaptoethanol. Therefore, PL3 was subjected to a second purification step by size-exclusion chromatography and all the experiments were performed under reducing conditions with protein fractions comprised in peaks 2–4.

Preliminary *in vitro* assays using radioactive pneumococcal cell walls or a suspension of R6 cells as substrates showed that purified PL3 displayed high murolytic and bactericidal activity and, thus, supported our hypothesis that PL3 could be a promising weapon against pneumococci. Therefore, its structural features, choline-binding affinity and structural stability were characterized.

Characterization of PL3 Structure: Effect of Choline Binding

Far- and Near-UV CD Spectra

The similarity of secondary and tertiary structures among the chimera PL3 and the parental enzymes was analyzed by CD. PL3 and Pal have very similar far-UV spectra (**Figure 3A**), as expected from their identical catalytic domain and linker and the high likeliness (77% sequence identity; 84% similarity) of their CBDs. Main differences found when compared with the LytA spectrum may be attributed to the different folds of their unrelated catalytic domains. Indeed, the two positive maxima displayed by PL3 and Pal spectra at 220–240 nm and the negative band at 200 nm (a shoulder in the Pal spectrum) likely correspond to a fingerprint of the *Amidase_5* domain. Choline binding strongly modified the far-UV spectra of PL3 and Pal; the intensity of the positive peak centered at 225–224 nm was almost doubled, whereas the negative band at 200 nm was highly reduced (the ellipticity became positive for the choline-bound form of Pal). The magnitude of such variations strongly contrasts with the rather local effect of choline binding on the LytA spectrum (the negative maximum at 225 nm became positive upon choline addition). In the near-UV region, largely dominated by the contributions of the CBD aromatic side-chains and sensitive to the tertiary and quaternary structures, the spectrum of unbound PL3 shows features of both parental enzymes, but it reminds the choline-bound spectra of LytA and Pal (**Figure 3B**). The likeness increased upon choline addition, but the intensity of spectrum of the choline-bound chimera was in between those of the parental enzymes.

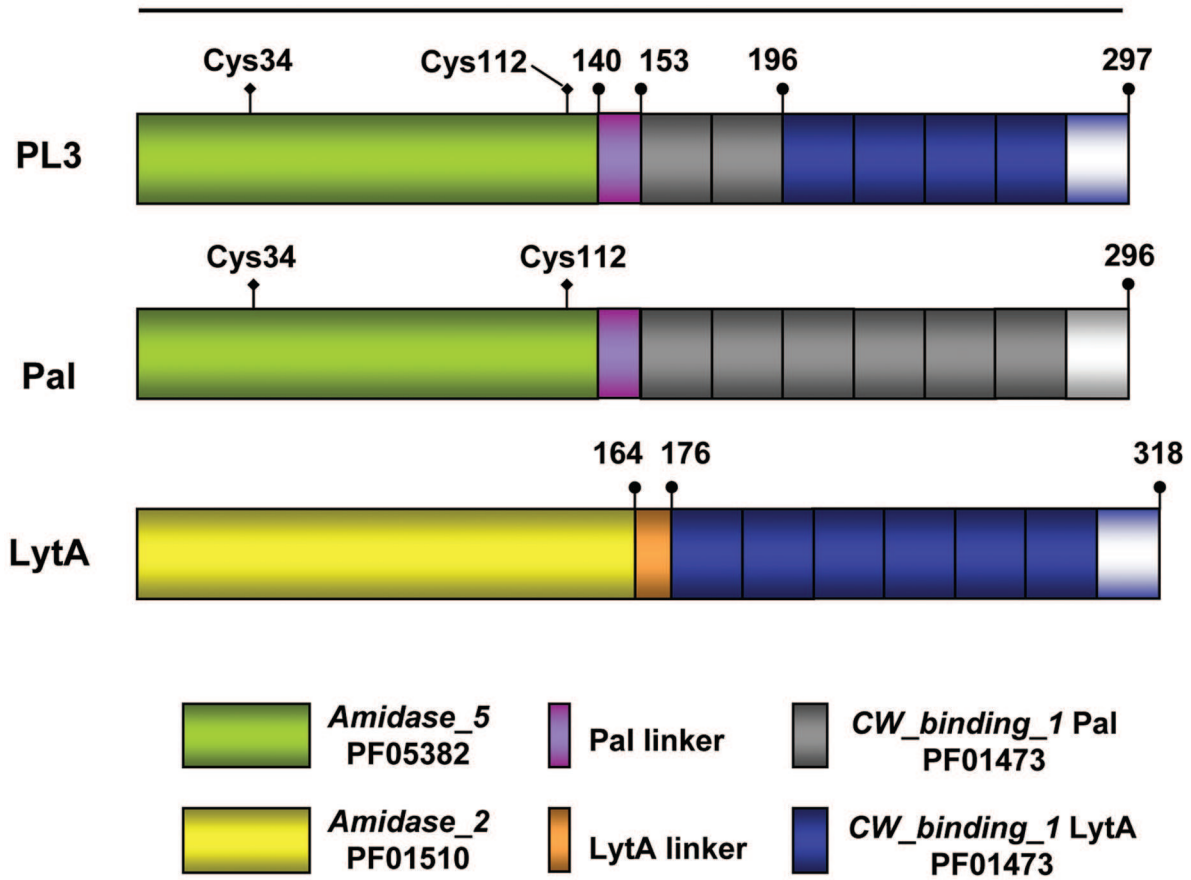

FIGURE 1 | Schematic representation of the PL3 chimeric NAM-amidase and the parental Pal and LytA murein hydrolases. Domain and linker origin is depicted by colors; gray and blue rectangles indicate the choline binding repeats comprised in Pal and LytA CBDs, respectively, followed by the C-terminal tail. Numbers show the end of domains and linkers. The position of the two cysteine residues in Pal and PL3 catalytic domains is marked. Pfam entries for *Amidase_2*, *Amidase_5*, and *CW_binding_1* (choline-binding repeats) families are also shown.

Modulation of PL3 Association States by Choline Binding

Like in other pneumococcal choline-binding proteins carrying a CBD composed of six choline-binding repeats and a C-terminal tail, choline binding regulates PL3 self-association, as depicted in **Figure 3C**. The distribution of sedimentation coefficients (c(s^*)) of the unbound protein showed a mayor peak with an $s_{20,w}$ of 6.8 S corresponding to the tetramer ($M_{w,app}$ = 130 kDa), a second peak compatible with the monomer ($s_{20,w}$ = 3.0; $M_{w,app}$ = 38 kDa) and a minor peak ($\leq$5% total area) at 10.0 S. Choline addition induced PL3 dimerization ($s_{20,w}$ = 4.1 S; $M_{w,app}$ = 64 kDa) with the subsequent reduction of monomer and tetramer populations. At 5 mM choline and higher, the dimer became the most favored form ($\approx$80% at 0.11 mg/ml PL3) although a small fraction of tetramer (10–12%) was still present. Stabilization of PL3 dimer was concomitant with the saturation of choline higher-affinity sites (see below), a feature shared with LytA, though the most populated form of the unbound autolysin was the dimer (Usobiaga et al., 1996; Varea et al., 2000). In contrast, the predominant species of the unbound and choline-bound forms of Pal were the monomer and the dimer, respectively, which coexisted, however, with lower fractions of higher association states (Varea et al., 2004). In addition, Pal dimerization was enhanced by saturation of the lower affinity sites (Varea et al., 2004). As shown in Supplementary Table S1, the sedimentation coefficients, the frictional coefficient ratios and Stokes radii calculated for a given state are almost identical in the three lysins, considering the higher molecular mass of the LytA monomer, which confirms that they have very similar hydrodynamic shapes.

CD Titration of PL3 with Choline

The titration curve of PL3, obtained by representing the relative variation in ellipticity at 295 nm as function of choline concentration (**Figure 3D**), presents two well defined phases, as in Pal and LytA (Medrano et al., 1996; Varea et al., 2004). Saturation of the higher affinity sites required lower choline concentration, compared to the parental enzymes, and correlated

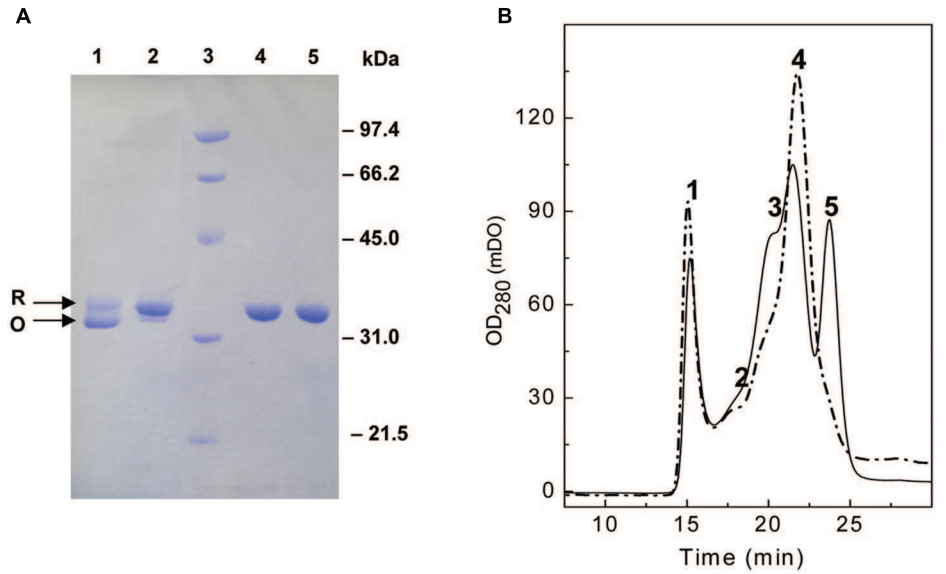

FIGURE 2 | Analysis of molecular forms of PL3. (A) Reducing and non-reducing SDS-PAGE profile of PL3. Protein equilibrated in PB or PB with 10 mM DTT was loaded in the gel using sample buffer without (lanes 1 and 2, respectively) or with (lines 4 and 5, respectively) β-mercaptoethanol. Lane 3, standard molecular mass markers. R and O arrows indicate bands corresponding to the reduced and oxidized form of PL3, respectively. **(B)** Size-exclusion fractioning of a PL3 sample, eluted from the DEAE-cellulose column, dialyzed against PB, and pre-equilibrated in a Superose 12 HR/10/30 column with PB, pH 6.8, containing (thick solid trace) or not (gray solid trace) 1 mM β-mercaptoethanol. Injection volume was 100 μl and the flow rate 0.5 ml/min. Large aggregates eluted in peak 1, whereas peak 5 likely corresponds to the oxidized monomer of PL3.

with PL3 dimerization. The apparent half-dissociation constants estimated through the description of PL3 titration profile in terms of two sigmoid functions were 60 ± 9 μM and 4.5 ± 0.6 mM for the higher and lower affinity sites, respectively, which are slightly lower than those estimated, with the same approach, for LytA (1.1 and 6.8 mM) and well below those of Pal (8 and 10 mM). In contrast, the cooperativity of choline binding to the lower affinity sites was much higher in the two parental enzymes (Supplementary Table S1).

Conformational Stability of PL3

Next, we analyzed the conformational stability of PL3 by DSC. The thermal denaturation curves registered in PB at pH 6.8 showed a mayor peak centered at 53.6°C with a shoulder at the lower temperature side (**Figure 4**). The shift of the major peak toward higher temperatures as choline concentration was increased allowed its assignment to the CBD. Moreover, choline-mediated stabilization eliminated the overlapping of the CBD and catalytic domain transitions, the latter becoming fully resolved with a transition temperature of 51°C in the presence of 20 mM choline. Like in Pal and LytA, the CBD stability increased at slightly basic pHs (Usobiaga et al., 1996; Varea et al., 2000, 2004) and was intermediate between those of the parental enzymes. As expected from LytA and Pal stability profiles (Varea et al., 2000, 2004), denaturation of the catalytic domain of PL3 begun around 10°C above that of LytA (Supplementary Figure S2).

Enzymatic Characteristics of PL3

Once characterized the biophysical properties related with the lytic activity and structural robustness of PL3, we examined its specific activity, very close to those of LytA and Pal (Supplementary Figure S1), and the optimal conditions to either degrade purified pneumococcal cell walls or kill R6 cells. Maximal activity was displayed in PB, 100 mM NaCl, 10 mM DTT, pH 6.8, for the two assays, the effective range of lysis extending from pH 6.5 to 8.0. These results were consistent with the optimal pH values reported for Pal (pH 6.9) and LytA (pH 6.8; Sheehan et al., 1997). The specific activity of PL3, 4×10^5 U/mg, was rather similar to that found for the parental proteins, Pal and LytA (Supplementary Figure S3). Remarkably, ≈95% of PL3 murolytic activity was maintained after been stored for 4 weeks at 37°C, and the bacteriolytic activity of the sample was fully preserved when tested against resuspended R6 cells. Moreover, lyophilization of PL3 did not provoke any loss of its murolytic and bacteriolytic capacity (data not shown).

Bactericidal Activity of PL3

First, the antibacterial capacity of PL3 was tested against several pneumococcal strains using the protocol described in the Section "Materials and Methods," which measures the turbidity decrease of bacterial suspensions and the cell survival after 60 min of incubation at 37°C, with and without the lysin. PL3 reduced the viability of all pneumococcal samples tested, including the multiresistant clinical strain 48 (serotype 23F), leading to the practical sterility of the cultures at concentrations of PL3 in the range of 0.5–5 μg/ml (**Figure 5**). In strong contrast with their similar lytic activities on purified cell walls (Supplementary Figure S1B), PL3 kills pneumococci more effectively than Pal and LytA, i.e., PL3 is capable to sterilize an R6 culture at 0.5 μg/ml, whereas Pal and LytA reduced 5 and 7.5 log units the viable

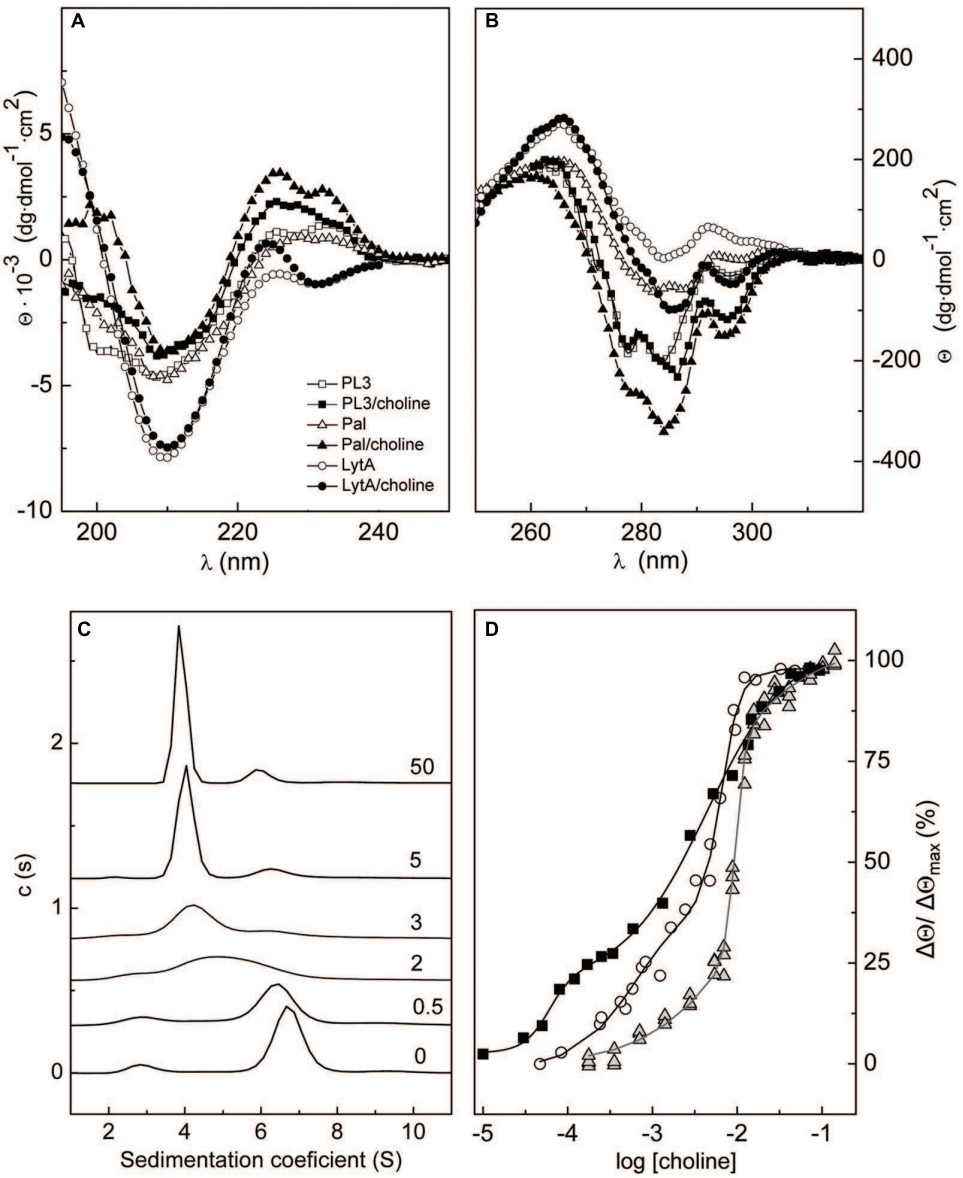

FIGURE 3 | Effect of choline binding on PL3 structure. Comparison of the far- **(A)** and near-UV **(B)** CD spectra of PL3, Pal, and LytA in the absence and in the presence of 20 mM choline. **(C)** Effect of choline binding on PL3 association-state distribution. Increase of choline concentration shifts the monomer/dimer/tetramer equilibria of PL3 toward the dimer ($s_{20,w}$ = 4.1 S) with the concomitant decrease of monomer ($s_{20,w}$ = 3.0) and tetramer ($s_{20,w}$ = 6.8 S) populations. Labels indicate the choline concentration (mM). **(D)** CD titration curve of PL3 (squares) generated from the ellipticity changes induced at 295 nm by choline binding. Titration curves of LytA (circles; Varea et al., 2000) and Pal (triangles; Varea et al., 2004) are also depicted. Continuous lines represent the fitting of experimental data as the sum of two sigmoids with the parameters shown in Supplementary Table S1.

cells, respectively, but at 10-fold higher concentration (5 μg/ml; Díez-Martínez et al., 2013). Moreover, at 0.1 μg/ml PL3 was even more lethal than Cpl-1 and Cpl-711 lysozymes against R6 and D39 strains, and as good as Cpl-711 against the multiresistant 48 strain (Díez-Martínez et al., 2015). It is worth noting that the type, thickness and net charge of the different capsular polysaccharides may somewhat modulate the lysin access to the peptidoglycan, as deduced from the higher susceptibility of R6 to the killing action of PL3 compared to that of the corresponding isogenic serotype-2 encapsulated D39 strain. This conclusion is further supported by the lower killing efficiency of PL3 obtained against P007, P008, and 48 strains, belonging to serotypes 3, 4, and 23F, respectively (**Figure 5**).

Since PL3 contains an specific CBD, it was feasible that this chimera could also lyse other Gram-positive pathogens apart from the pneumococcus, provided that they contain choline in the (lipo)teichoic acids. Thus, the bactericidal activity of PL3 was tested on various non-pneumococcal streptococci. As shown in **Figure 6**, PL3 efficiently killed *S. oralis*, *S. pseudopneumoniae*, and *S. mitis* type strains (harboring choline in their (lipo)teichoic

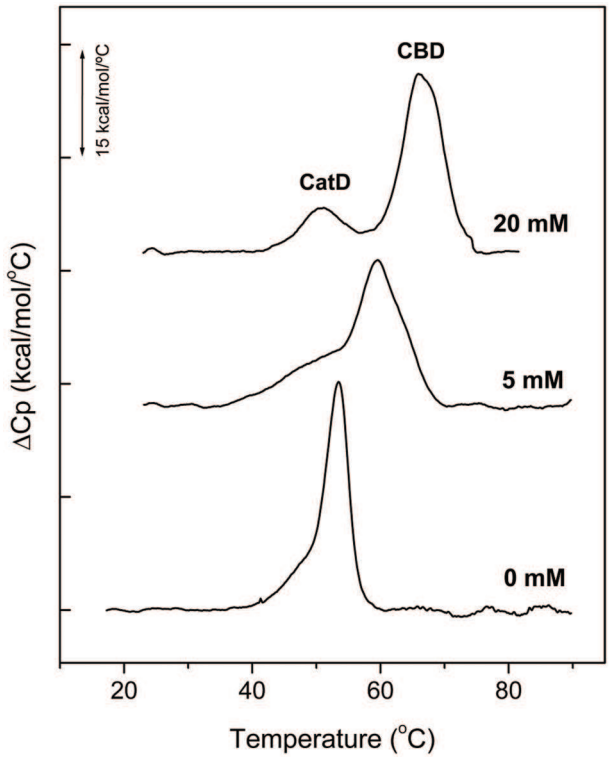

FIGURE 4 | Thermal denaturation curves of PL3. DSC profiles of PL3 were measured in the absence and in the presence of choline at ligand concentrations specified in figure labels. Experiments were performed at 0.4 mg/ml PL3 in PB, pH 6.8 (scan rate 60°C/h). The curves were shifted along the *y*-axis for clarity. CatD and CBD indicate the transitions corresponding to the catalytic domain and the cell wall-binding domain, respectively.

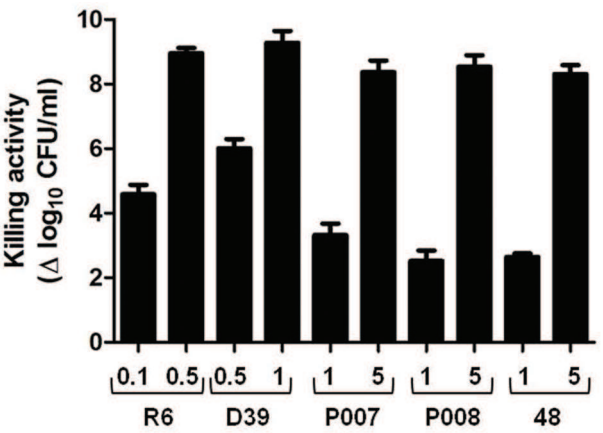

FIGURE 5 | Bactericidal activity of PL3 against pneumococcal strains. Exponentially growing cultures were washed, resuspended in PB containing 100 mM NaCl and 10 mM DTT (pH 6.8), and treated with different concentrations of PL3 for 60 min at 37°C. Then, viable cells were measured and killing value was calculated as the decrease of bacterial titers, in log units, compared to PL3-untreated control. Protein concentrations (μg/ml) are indicated in the *x*-axis. Error bars represent standard deviations and differences in activity values are statistically significant ($P < 0.05$).

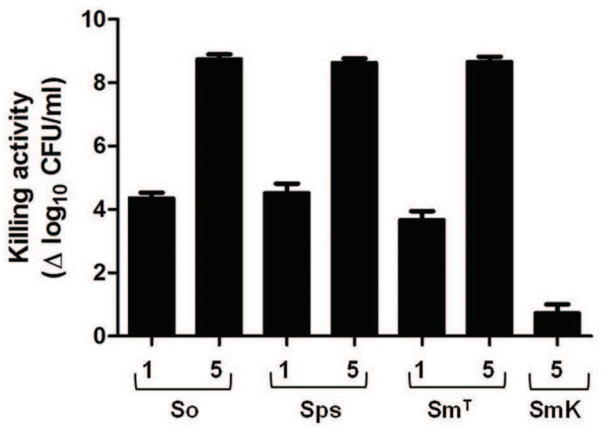

FIGURE 6 | Bactericidal activity of PL3 against non-pneumococcal species. The assay was performed as described in **Figure 5**. Numbers on *x*-axis indicate the concentration of PL3, in μg/ml. So, *S. oralis*; Sps, *S. pseudopneumoniae*; Sm^T, *S. mitis* type strain; SmK, *S. mitis* SK598 strain. Error bars represent standard deviations and differences in activity values are statistically significant ($P < 0.05$).

acids), although to eradicate the cells down to the limit of detection it was necessary to add the enzyme at 5 μg/ml. As expected, *S. mitis* SK598 strain, which possesses ethanolamine-containing teichoic acids (Bergström et al., 2003), was refractory to PL3 bacteriolytic action.

Effect of the Addition of PL3 to Different Phases of the Growth Curve

After studying the exogenous bacteriolytic action of PL3 on bacterial suspensions, we also investigated its effect along the growing curve of R6 and the isogenic strain P046, a double mutant lacking LytC and LytA autolysins, whose comparison would allow evaluating the influence of endogenous autolysins in PL3-mediated bacteriolysis. Thus, we added the same concentration of PL3 (2.7 μg/ml) to different cultures of R6 or P046 at early exponential ($OD_{550} \approx 0.15$), late exponential ($OD_{550} \approx 0.4$) and stationary phase of growth ($OD_{550} \approx 0.7$) and the turbidity decrease was monitored at 37°C. Viable cells were also determined 3.5 h after culture initiation. The addition of PL3 at any phase of the growth curve produced an immediate and marked OD_{550} decrease, with the concomitant efficient killing of both R6 and P046 cells (**Figures 7A,B**). Notably, the bactericidal effect depended on the strain and the growth phase; whereas PL3 addition sterilized R6 cultures at any point of the growing curve, the number of viable cells for late exponential or stationary P046 cultures was reduced only 2 logs (**Figure 7C**). This difference strongly suggests a synergistic effect between endogenous LytA and LytC autolysins and exogenous PL3 to trigger cell lysis. It is also noteworthy that when the experiment was carried out without DTT in the media, the bacterial population can resume growing again after an initial decrease in the OD_{550} and cell survival was significantly increased, thereby demonstrating that

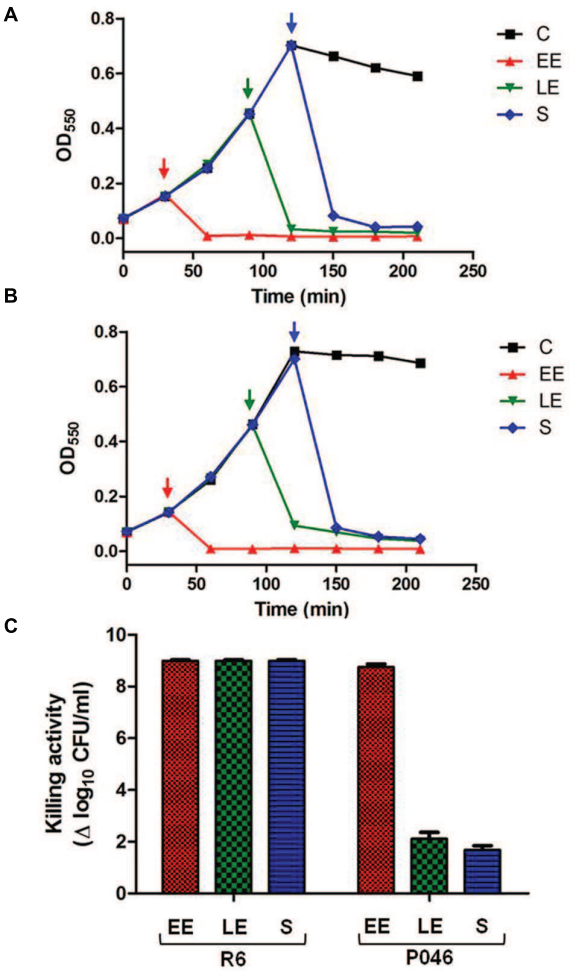

FIGURE 7 | Effect of PL3 on growth of pneumococcal R6 and P046 cultures. (A,B) R6 and P046 growing curves where 2.7 μg/ml PL3 were added at different phases of growth (marked by arrows) in the presence of 10 mM DTT. **(C)** Log of killed cells was calculated by comparing CFUs of treated- and PL3-untreated cultures determined 210 min after culture initiation. EE, early exponential phase; LE, late exponential phase; S, stationary phase; C, untreated control. Error bars represent standard deviations and differences in activity values are statistically significant ($P < 0.05$).

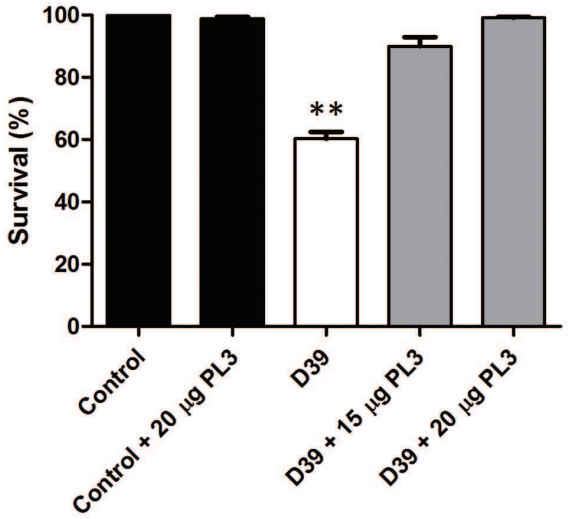

FIGURE 8 | Rescue of zebrafish embryos from pneumococcal infection by PL3. Survival of embryos infected with D39 *S. pneumoniae* strain treated or not with single doses of PL3. Data are means from four independent experiments. Error bars represent standard deviation and asterisks mark the results that are statistically significant for the overall comparison of infected or PL3-treated embryos *vs.* the controls (one-way ANOVA with a *post hoc* Dunnett test; $^{**}P < 0.001$).

reducing conditions were necessary for the full activity of PL3 (data not shown).

Bactericidal Activity of PL3 in an Infection Animal Model

The experiments described above proved that PL3 efficiently kills pneumococci and relative bacteria *in vitro*. To validate these data *in vivo*, we used the already set up zebrafish embryo model for studying anti-streptococcal compounds (Díez-Martínez et al., 2013). Zebrafish embryos were brought in contact with the pneumococcal strain D39 by immersion in E3 medium containing 1 mM DTT and incubated for 8 h at 28°C, using heat-killed D39 cells (10 min at 65°C) as negative controls. Afterward, embryos were extensively washed with E3 medium and treated with different amounts of PL3, or the corresponding volume of the enzyme buffer, as described in the Section "Materials and Methods." The mortality rate of infected embryos was around 40%, associated with inflammation of heart and liver, and death occurred at about 48–72 h post-infection. Addition of a single 20-μg dose of PL3 to pneumococcus-infected embryos fully protected them from death, whereas survival dropped to about 90% when treated with 15 μg (**Figure 8**). The level of protection provided by PL3 was higher than those of Cpl-1 or Cpl-7S whose complete protection of embryos was achieved at a dose of 25 μg (Díez-Martínez et al., 2013).

DISCUSSION

The increased incidence of antibiotic resistance has led to a renewed search for novel antimicrobials. In this context, the use of pathogen-directed antibacterials through the employ of specific lytic peptidoglycan hydrolases appears as an alternative to diminish the rate of antibiotic-resistant pathogens worldwide (Czaplewski et al., 2016). Most lysins reported as effective antibacterials so far have a phage origin, encoded by lytic (virulent) or temperate phages. Besides these "natural" genes as source for lysins, another strategy is to combine catalytic and substrate-binding domains from different origins to construct fusion enzymes with novel bactericidal properties or enhanced activity, solubility or stability (Donovan et al., 2006; Daniel et al., 2010; Díez-Martínez et al., 2015; Yang et al., 2015). Knowledge of the structural and functional properties of selected domains and their compatibility with the envelope structure of the targeted bacterium are key factors

to successfully develop tailor-made lysins. Here we describe the design, production and characterization of PL3, a robust chimeric NAM-amidase constructed by substitution of the last four choline binding repeats and the C-terminal tail of the Pal endolysin by those of LytA NAM-amidase, the major pneumococcal autolysin. Remarkably, PL3 bacteriolytic activity from the outside goes beyond those of the parental lysins against all the pneumococcal clones tested, including the 48 strain, which is resistant to penicillin, erythromycin, tetracycline, and quinolones. Depending on the strain, PL3 compares, and even surpasses, the bacteriolytic activity of Cpl-711, the more effective enzybiotic against *S. pneumoniae* so far described (Díez-Martínez et al., 2015). In contrast with some antibiotics, and like other lysins, PL3 also has the advantage of being effective against targeted bacteria at any metabolic state of the culture. In particular, PL3 was able to kill bacteria at the stationary phase of growth when the metabolic machinery, i.e., the level of protein synthesis, was notably reduced. The antimicrobial efficacy of PL3 has been also validated *in vivo* using a zebrafish embryo infection model, where a single dose of 20 μg fully protects against death by infection with pneumococcal D39 strain.

PL3 exhibits also a potent lytic activity against *S. pseudopneumoniae*, *S. oralis*, and *S. mitis* strains that contain choline in the cell wall, which opens up its use to treat the infections caused by these opportunistic pathogens. Namely, *S. pseudopneumoniae* may cause pneumonia, bronchitis and chronic sinusitis with greater resistance than pneumococcus to several antimicrobials (Laurens et al., 2012). *S. mitis* can cause a broad range of infections from caries to invasive diseases like endocarditis, bacteremia, pneumonia, etc., with resistance to common antibiotics (Mitchell, 2011). It is also an emergent causative of blood infections in immunocompromised patients (Shelburne et al., 2014) and has been associated to the toxic shock syndrome with mortality rates above 60% (Tunkel and Sepkowitz, 2002). Finally, *S. oralis* may produce bacterial endocarditis, respiratory distress syndrome and streptococcal shock in immunocompromised individuals (Verhagen et al., 2006). It is also involved in periodontal disease, the most common infection of the human oral cavity (Maeda et al., 2013). In this respect, PL3 is the first lysin reported to effectively kill *S. oralis*.

As expected from previous studies (Usobiaga et al., 1996; Varea et al., 2004), modification of the CBD of Pal leading to the PL3 chimera drastically enhanced choline binding and reduced the concentration required for choline-induced dimerization, two factors shown to be essential for the activity (Varea et al., 2000), without perceptibly affecting the shape of the chimera in relation to the equivalent association state of Pal or LytA. Moreover, saturation of the higher affinity sites occurs in PL3 at lower choline concentration than in LytA, and these features could explain, at least partially, the remarkable superiority of PL3 in eradicating *S. pneumoniae*. These results might also mean that the site located at the interface of the second and third repeat, different in PL3, LytA and Pal, might be central in choline recognition by the CBD. On the other hand, the reduction of PL3 negative net charge in three units with respect to LytA may further increase the bactericidal activity, by facilitating the accessibility of PL3 to the peptidoglycan network through the negatively charged outside of the bacteria (Low et al., 2011; Díez-Martínez et al., 2013). Such effect might also explain why the chimera and the parental lysins display highly different relative activities when tested on bacterial suspensions and very similar on purified pneumococcal cell walls, where substrate fragmentation facilitates the accessibility and cleavage of susceptible bonds.

Domain interchange conferred a remarkable conformational robustness to PL3, evidenced by preservation of around 95% activity when tested on purified pneumococcal cell walls or bacterial suspension after been stored 4 weeks at 37°C. Notably, PL3 can even be lyophilized without any loss of activity when assayed against the two types of substrates. Other interesting finding of this study is the apparent cooperation of bacterial autolysins to the exogenous bacteriolytic action of PL3, which reminds the combined action of LytA and LytC with CbpD in pneumococcal fratricide (Eldholm et al., 2009), and may open new clues about the mechanism of action of lysins as anti-infectives. Specifically, the absence of endogenous LytA and LytC autolysins in pneumococcal P046 strain reduced by around 7 log units the number of killed bacteria in relation with the isogenic R6 strain, when exponential and stationary phase grown cultures were treated with PL3.

Current evolution of the clinical trials is responding to the initial hope of lysins as effective and specific alternative antibacterials to fight most dangerous and multiresistant pathogens. Indeed, the first commercial lysin is already on the market for topical treatment of skin infections provoked by methicillin-resistant *Staphylococcus aureus* (MRSA)[2]. Furthermore, according to the portfolios of several pharmaceutical companies, this example is expected to be followed by other lysins in the near future. The results described in this work show that structure and function-based approach to construct tailor-made lysins by domain shuffling from parental proteins is an advantageous alternative and it could be a general method to design a 'magic bullet' directed against selected pathogens, provided that in-depth knowledge on the enzymes and substrate characteristics are in hand. In this sense, PL3 could be an appropriate candidate, alone or in combination with other active lysins, for the toolbox to combat multiresistant infections provoked by any pneumococcal strain or closely related pathogens.

CONCLUSION

We report here the design, production, and characterization of PL3, a chimeric lysin with a robust bacteriolytic activity *in vivo* and *in vitro* against *S. pneumoniae* and other streptococci bearing choline in the cell wall. Due to its remarkable stability, lyophilization feasibility, and killing efficiency at very low doses, PL3 has a great potential to be used as an effective therapeutic agent against susceptible and multiresistant pathogens.

[2]http://www.micreos.com/

AUTHOR CONTRIBUTIONS

BB, AF-T, MI-B, MM, and PG designed the experiments, performed by BB, AF-T, and MI-B. BB, AF-T, MI-B, MM, and PG discussed the results. MM and PG supervised the study and wrote the manuscript with contributions of BB, AF-T, and MI-B. All authors read, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01156

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Antibacterial Activity of a Novel Peptide-Modified Lysin Against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

*Hang Yang†, Mengyue Wang†, Junping Yu and Hongping Wei**

Key Laboratory of Special Pathogens and Biosafety, Center for Emerging Infectious Diseases, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China

The global emergence of multidrug-resistant (MDR) bacteria is a growing threat to public health worldwide. Natural bacteriophage lysins are promising alternatives in the treatment of infections caused by Gram-positive pathogens, but not Gram-negative ones, like *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, due to the barriers posed by their outer membranes. Recently, modifying a natural lysin with an antimicrobial peptide was found able to break the barriers, and to kill Gram-negative pathogens. Herein, a new peptide-modified lysin (PlyA) was constructed by fusing the cecropin A peptide residues 1–8 (KWKLFKKI) with the OBPgp279 lysin and its antibacterial activity was studied. PlyA showed good and broad antibacterial activities against logarithmic phase *A. baumannii* and *P. aeruginosa*, but much reduced activities against the cells in stationary phase. Addition of outer membrane permeabilizers (EDTA and citric acid) could enhance the antibacterial activity of PlyA against stationary phase cells. Finally, no antibacterial activity of PlyA could be observed in some bio-matrices, such as culture media, milk, and sera. In conclusion, we reported here a novel peptide-modified lysin with significant antibacterial activity against both logarithmic (without OMPs) and stationary phase (with OMPs) *A. baumannii* and *P. aeruginosa* cells in buffer, but further optimization is needed to achieve broad activity in diverse bio-matrices.

Keywords: bacteriophage lysin, engineered lysin, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, outer membrane permeabilizers (OMPs)

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Yves Briers, Ghent University, Belgium

****Correspondence:***
Hongping Wei
hpwei@wh.iov.cn

†These authors have contributed equally to this work.

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INTRODUCTION

The global emergence of multidrug-resistant (MDR) Gram-negative bacteria is a growing threat to public health worldwide (Li et al., 2015). Due to the diversity of resistance mechanisms that may lead to MDR or even pandrug resistance (PDR; Livermore and Woodford, 2006; Potron et al., 2015), *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are among the increasingly reported and commonly identified MDR or even PDR nosocomial pathogens. They are responsible for many hospital-acquired infections, ranging from mild skin wounds and urinary tract infections to severe life-threatening infections, including bloodstream, pneumonia, and meningitis (Garcia-Quintanilla et al., 2013; Bassetti et al., 2014). At the same time, the progress in developing new antibiotics against these pathogens is slow (Fischbach and Walsh, 2009). Therefore, new strategies for controlling these MDR pathogens are urgently needed.

Bacteriophage lysins, the weapon of phages to digest the host bacterial cell wall for the release of progeny phages, have been extensively demonstrated to be promising alternatives in the treatment of Gram-positive pathogens, such as staphylococci and streptococci (Nelson et al., 2012; Pastagia et al., 2013; Yang et al., 2014). Due to their unique working mechanisms, lysins possess a low possibility of developing resistance (Fischetti, 2008; Knoll and Mylonakis, 2014). However, the outer membranes of Gram-negative bacteria block the access of natural lysins to their peptidoglycan substrates, thus making the exogenously added lysins useless or very weak against the viability of the target cells (Morita et al., 2001; Lai et al., 2011; Lim et al., 2014).

Currently, several strategies have been developed to break the barriers posed by the outer membranes of Gram-negative bacteria to natural lysins. Physical (i.e., high hydrostatic pressure; Briers et al., 2008) and chemical permeabilizers (i.e., EDTA, and weak organic acid, usually citric acid; Briers et al., 2007, 2011) can permeabilize the outer membrane efficiently to enhance the antibacterial activity of lysins, but are only applicable in applications such as food conservation and the treatment of topical infections. Structure-based engineering and phage genome-based screening methods have also been used to find novel lysins that act on *Yersinia* with the FyuA receptor (Lukacik et al., 2012) and *A. baumannii* (Lood et al., 2015). In recent years, a few engineered lysins have been reported with good antibacterial activities against *P. aeruginosa* by fusing natural lysins with optimized N- or C-terminal lipopolysaccharides-destabilizing or antimicrobial peptides, respectively, which can permeabilize the outer membranes (Briers et al., 2014a,b). In principle, considering the easy availability of lipopolysaccharide-destabilizing and antimicrobial peptides, fusing natural lysins with such peptides looks quite attractive since it provides a good way with plenty of chances to create novel engineered lysins against Gram-negative bacteria. However, reports on peptide-modified lysins are limited, and mainly focus on *P. aeruginosa*.

In the present study, a new peptide-modified lysin (PlyA) against *A. baumannii* and *P. aeruginosa* was constructed by fusing the cecropin A peptide residues 1–8 (KWKLFKKI) with the OBPgp279 lysin (Walmagh et al., 2012), and its antibacterial activity was evaluated.

MATERIALS AND METHODS

Bacterial Strains

All bacterial strains and clinical isolates (**Table 1**) used in this work were grown in Luria Broth (LB) medium at 37°C. All clinical isolates of *A. baumannii* and *P. aeruginosa* were identified by 16S rDNA sequencing analysis combined with the biochemistry test using a MicroStation system (Biolog, GEN III Omnilog Combo Plus System, USA).

Construction of Plasmids

Cecropin A is a 37-residue membrane-active antimicrobial polypeptide that kills bacteria by dissipating transmembrane electrochemical ion-gradients (Silvestro and Axelsen, 2000). Because the N-terminal residues 1–8 of cecropin A (CA, KWKLFKKI) are highly positively charged, this fragment was used in this study. OBPgp279 coding sequence was initially cloned into the modified pET28a(+) plasmid (Kan^R) containing a $(G4S)_2$ liner between *Bam*HI and *Eco*RI sites (Yang et al., 2012), using primers OBP-F (5-tatagaattcatgaaaaactcggaaaagaacg-3) and OBP-R (5-atatctcgagcacgatacccagagctttttg-3), to obtain pET-*OBPgp279* vector (Kan^R). The coding sequence for cecropin A peptide residues 1–8 (CA, KWKLFKKI) was cloned into the pET-*OBPgp279* vector, using primers CA-F (5-catgggcaaatggaaattatttaagaaaattg-3) and CA-R (5-gatccaattttcttaaataatttccatttgcc-3), to obtain pET-CA-*OBPgp279* (pET-PlyA, Kan^R) vector. After verification by sequencing, *E. coli* BL21(DE3) cells were transformed with the correct plasmid for protein expression.

Protein Purification

The recombinant enzymes were purified as described previously in our laboratory (Huang et al., 2015). Briefly, the *E. coli* BL21(DE3) cells were induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) overnight at 16°C and collected for protein purification after sonication on ice. Then the proteins were collected by washing and eluting with 80 and 400 mM imidazole through a nickel nitrilotriacetic acid column (GE Healthcare, US), respectively. The collected active protein fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 7.4) and stored at –80°C until use (less than 2 weeks).

Antibacterial Activity Assay

To determine the antibacterial activity of PlyA, logarithmic (cultured for 3–4 h, OD_{600} = 0.6–0.8) or stationary phase (cultured for 14–16 h, OD_{600} = 1.4–1.6) cultures of *A. baumannii* WHG3066 were centrifuged (10,000 $g \times 1$ min) first. Then the cells were washed once and resuspended in 20 mM Tris-HCl (pH 7.4). Bacterial suspensions (100 μl) were mixed with the enzyme in the presence or absence of the outer membrane permeabilizers (OMPs, i.e., EDTA and citric acid) at 37°C for 15–60 min. Finally, the remaining viable cells were calculated by plating onto LA plates. For susceptibility test, clinical isolates of *A. baumannii* and *P. aeruginosa* were cultured to logarithmic phase and treated with 50 (for *A. baumannii* isolates) or 100 μg/ml (for *P. aeruginosa* isolates) PlyA at 37°C for 1 h. All assays were performed for at least three times in biological repeats.

To test the synergism between OMPs and PlyA, stationary phase *A. baumannii* WHG3066 and *P. aeruginosa* WHG3012 cells were treated with 100 μg/ml PlyA in the presence of various concentrations of EDTA or citric acid in 20 mM Tris-HCl (pH 7.4) or 5 mM HEPES-NaOH (pH 7.4) at 37°C for 1 h. Afterward, the viable cell numbers were counted by plating. To avoid the acidification effect of citric acid, the synergism between citric acid and PlyA was also performed by adjusting the pH values of the reaction systems to 7.4. All assays were performed for at least three times in biological repeats.

To test the effects of bio-matrix on the antibacterial activity of PlyA, *A. baumannii* WHG3066 cells in logarithmic phase were washed once with 20 mM Tris-HCl buffer (pH 7.4), and resuspended in medium, including LB, Mueller–Hinton (MH, Huankai Microbial, Guangdong, China), Brain Heart Infusion

TABLE 1 | Bacterial strains and clinical isolates used in this work.

Strain	Antibiogram	Strain	Antibiogram
Acinetobacter baumannii[a]		***Pseudomonas aeruginosa***[a]	
WHG3047	ND	WHG3012	Ak^S, Ge^S, Cf^S, Im^S
WHG3051	ND	WHG3022	Ak^S, Ge^R, Cf^R, Im^R
WHG3059	ND	WHG3029	Ak^S, Ge^S, Cf^S, Im^S
WHG3072	Ak^R, Ge^R, Cf^R, Im^R	WHG3014	Ak^S, Ge^S, Cf^S, Im^S
WHG3074	Ak^R, Ge^R, Cf^R, Im^R	WHG3015	Ak^S, Ge^S, Cf^R, Im^S
WHG3075	Ak^R, Cf^R, Im^R	WHG3028	Ak^S, Ge^S, Cf^R, Im^R
WHG3032	Ak^S, Ge^S, Cf^S, Im^S	WHG3030	Ak^S, Cf^S, Im^S
WHG3064	Ak^S, Ge^S, Cf^S, Im^S	WHG3013	Ak^R, Ge^R, Cf^R, Im^R
WHG3078	Ak^R, Ge^R, Cf^R, Im^R	WHG3043	Ak^S, Ge^S, Cf^S, Im^R
WHG3048	ND	WHG3006	Ak^S, Ge^S, Cf^S, Im^S
WHG3049	ND	WHG3007	Ak^S, Ge^S, Cf^S, Im^R
WHG3050	ND	WHG3008	Ak^S, Ge^S, Cf^S, Im^R
WHG3052	ND	WHG3009	Ak^S, Ge^S, Cf^S, Im^R
WHG3053	ND	WHG3016	Ak^S, Ge^S, Cf^S, Im^I
WHG3054	ND	WHG3018	Ak^S, Ge^S, Cf^S, Im^S
WHG3082	Ak^S, Ge^S, Cf^S, Im^S	WHG3021	Ak^S, Ge^S, Cf^S, Im^R
WHG3073	Ak^R, Ge^R, Cf^R, Im^R	WHG3019	Ak^S, Ge^S, Cf^R, Im^R
WHG3070	Ak^R, Ge^R, Cf^R, Im^R	WHG3024	Ak^R, Ge^R, Cf^R, Im^R
WHG3071	Ak^R, Ge^R, Cf^R, Im^R	WHG3025	Ak^S, Ge^S, Cf^S, Im^R
WHG3077	Ak^R, Ge^R, Cf^R, Im^R	WHG3026	Ak^S, Ge^S, Cf^S, Im^S
WHG3083	Ak^R, Ge^R, Cf^R, Im^R	WHG3031	Ak^R, Ge^R, Cf^R, Im^R
WHG3079	Ak^R, Ge^R, Cf^R, Im^R	WHG3033	Ak^S, Ge^S, Cf^S, Im^R
WHG3080	Ak^R, Ge^R, Cf^R, Im^R	WHG3034	Ak^S, Ge^S, Cf^S, Im^S
WHG3081	Ak^R, Ge^R, Cf^R, Im^R	WHG3036	Ak^S, Ge^S, Cf^S, Im^S
WHG3056	Ak^S, Ge^S, Cf^S, Im^S	WHG3017	Ak^S, Ge^S, Cf^S, Im^S
WHG3058	Ak^R, Ge^R, Cf^R, Im^R	WHG3037	Ak^S, Ge^S, Cf^S, Im^S
WHG3061	Ak^R, Ge^R, Cf^R, Im^R	WHG3039	Ak^S, Ge^R, Cf^S, Im^S
WHG3063	Ak^S, Ge^R, Cf^R, Im^R	WHG3038	Ak^R, Ge^R, Cf^R, Im^R
WHG3062	Ak^R, Ge^R, Cf^R, Im^S	WHG3040	Ak^S, Ge^S, Cf^I, Im^S
WHG3065	Ak^R, Ge^R, Cf^R, Im^R	WHG3041	Ak^S, Ge^S, Cf^S, Im^S
WHG3068	Ak^S, Ge^R, Cf^R, Im^R	WHG3042	Ak^S, Ge^S, Cf^S, Im^S
WHG3066	Ak^S, Ge^S, Cf^R, Im^R	WHG3023	Ak^S, Ge^S, Cf^S, Im^S
Escherichia coli[b]		WHG3027	Ak^S, Ge^S, Cf^R, Im^R
BL21(DE3)	ND	WHG3029	Ak^S, Ge^S, Cf^S, Im^S
Bacillus cereus[b]		WHG3014	Ak^S, Ge^S, Cf^S, Im^S
IS195	ND	WHG3015	Ak^R, Ge^R, Cf^S, Im^R

Antibiogram: Ak, amikacin; Ge, gentamicin; Cf, cefepime; Im, imipenem; ND, not detected; R, resistant; S, susceptible; I, intermediate-resistant.
[a]*Isolated from Zhongnan Hospital of Wuhan University;* [b]*Lab collection.*

(BHI, Huankai Microbial, Guangdong, China) and Tryptic Soy Broth (TSB, Becton, Dickinson & Co., France) with 4% NaCl (TSBN), or pasteurized milk (Mengniu Group, Wuhan, China), or human serum (Sigma–Aldrich, Shanghai, China). Then, cells were treated with 100 μg/ml PlyA at 37°C for 1 h, respectively. The viable cell numbers were evaluated by plating on LA plates. All assays were performed for at least three times in biological repeats.

Transmission Electron Microscope (TEM)

The action of PlyA on the cell wall of the bacteria was monitored by a thin-section transmission electron microscope (Tecnai G^2 20 TWIN, FEI, USA). Briefly, *A. baumannii* WHG3066 cell suspensions in logarithmic phase were incubated with 100 μg/ml PlyA at 37°C for 10, 15, and 30 min, respectively. Then, the reactions were terminated by addition of 2.5% glutaraldehyde and the fixed samples were analyzed by transmission electron microscope (TEM). Cells treated with 20 mM Tris-HCl (pH 7.4) under the same conditions were used as controls.

RESULTS

Characteristics of PlyA in Tris-HCl Buffer

As shown in **Figure 1A**, CA-fused OBPgp279 (called PlyA for short) and its parental lysin OBPgp279 were well expressed in *E. coli* as soluble proteins with purity of >95% as observed by 12% SDS-PAGE gel. Antibacterial activity tests showed that

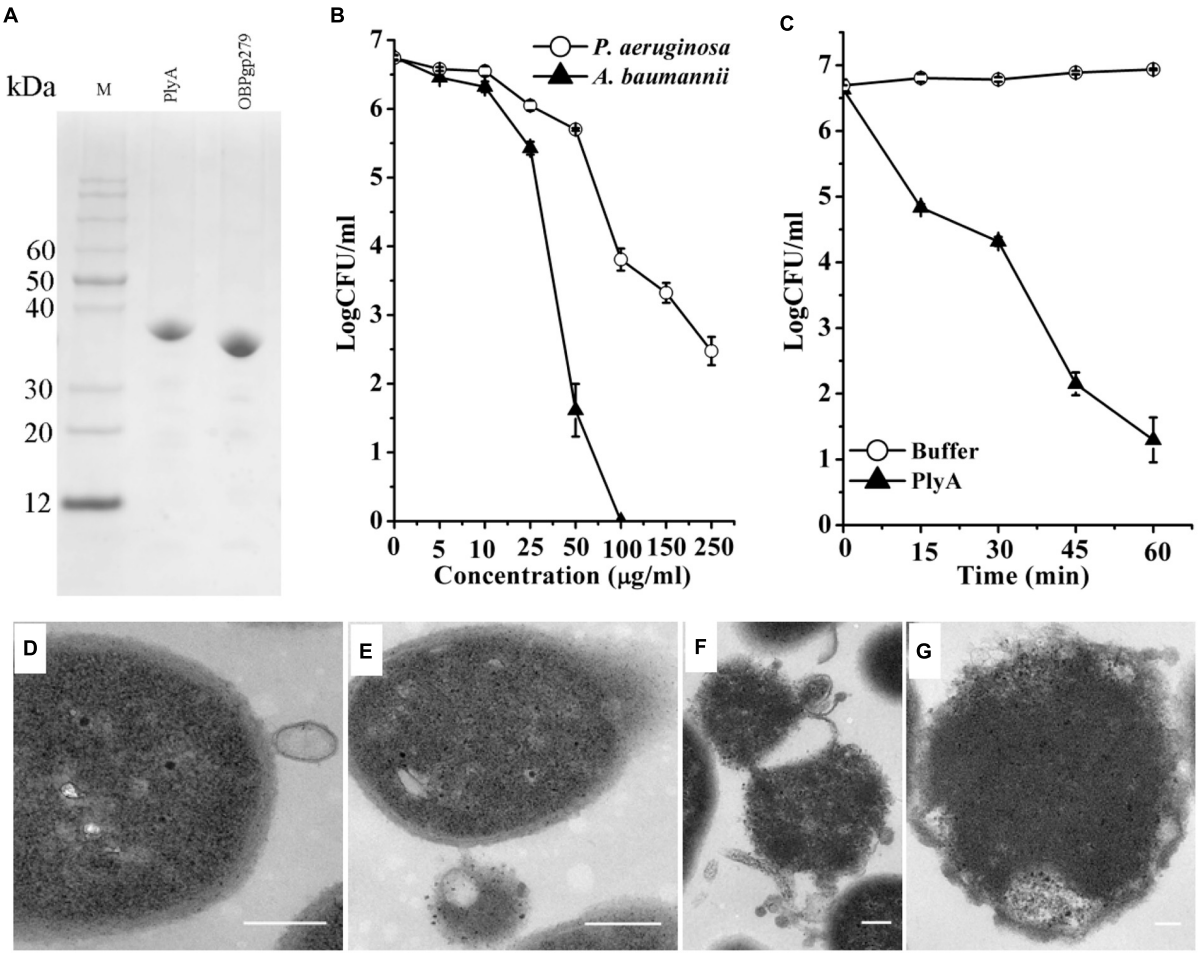

FIGURE 1 | Activity of peptide-modified lysin (PlyA) in Tris-HCl buffer. (A) Analysis of purified proteins on 12% SDS-PAGE gel. **(B)** Dose-dependent antibacterial activity of PlyA against logarithmic *Acinetobacter baumannii* WHG3066 and *Pseudomonas aeruginosa* WHG3012 in 20 mM Tris-HCl (pH 7.4). **(C)** Time-killing curve of 50 μg/ml PlyA against logarithmic *A. baumannii* WHG3066. **(D–G)** Transmission electron microscope (TEM) images of logarithmic *A. baumannii* WHG3066 exposed to PlyA. TEM analysis revealed that *A. baumannii* cells exposed to 100 μg/ml PlyA suffered from extrusion **(D)** and rapid disruption at single **(E)** or multiple sites **(F)**, resulting in the loss of cytoplasmic contents and ultimately the formation of ghost cell **(G)**. Bar sizes: 200 nm.

PlyA could kill logarithmic phase *A. baumannii* WHG3066 and *P. aeruginosa* WHG3012 cells rapidly in a dose-dependent manner (**Figure 1B**). Specifically, a reduction of over 5 logs (from 6.6 log to 1.2 log) was observed after treating the *A. baumannii* cells with 50 μg/ml PlyA for 1 h (**Figure 1B**), and a reduction about 2.5 logs (from 6.7 log to 3.8 log at 100 μg/ml PlyA) for *P. aeruginosa* cells (**Figure 1B**). Nearly no viable *A. baumannii* cells were detected when the concentration of PlyA increased to 100 μg/ml. While its parental lysin OBPgp279 (with equimolar) could only cause a reduction of about 1.38 logs (data not shown). The time kill curve revealed that the viable *A. baumannii* cell numbers were reduced about 1.8 logs (from 6.6 log to 4.8 log) within the first 15 min when treated with 50 μg/ml PlyA, and the killing continued for at least 60 min (**Figure 1C**).

Transmission electron microscope Images revealed that the *A. baumannii* WHG3066 cells exposed to PlyA suffered from leakage (**Figure 1D**) and rapid disruption at single (**Figure 1E**) or multiple sites (**Figure 1F**), resulting in the partial or total loss of cytoplasmic contents and ultimately loss of cell integrity (**Figure 1G**).

Antibacterial Spectrum of PlyA

Next, we tested the susceptibility of a collection of clinical isolates of *A. baumannii* and *P. aeruginosa* to PlyA (in Tris-HCl), including another 31 *A. baumannii* isolates and 35 *P. aeruginosa* isolates with multiple antimicrobial resistances profiles (**Table 1**). The plating assay revealed that PlyA was active against all *A. baumannii* isolates tested, causing a reduction of 1.0–4.2 logs. Except WHG3033, all *P. aeruginosa* isolates tested (35/36) were susceptible to PlyA (with a reduction of 0.5–2.7 logs in viable cell number; **Figure 2**). The variable susceptibility of these clinical isolates to PlyA may be due to their different modifications in their outer membrane structure. No antibacterial activity of PlyA was observed against *Escherichia coli* BL21(DE3) and *Bacillus cereus* IS195 tested (data not shown). These results

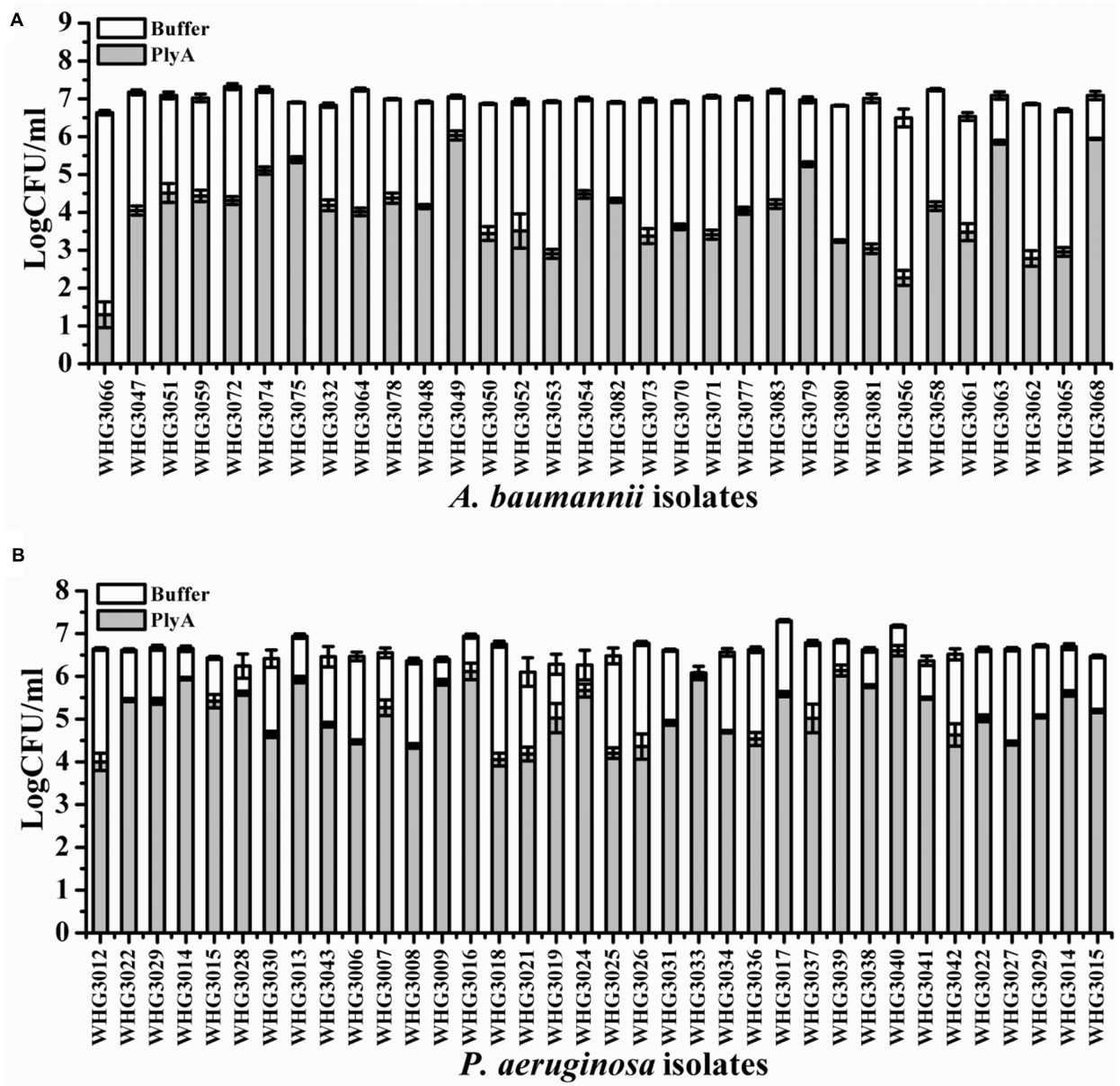

FIGURE 2 | Antibacterial activity of PlyA against clinical isolates of *A. baumannii* and *P. aeruginosa* in 20 mM Tris-HCl (pH 7.4). (A) Antibacterial activity of PlyA (50 μg/ml) against *A. baumannii* clinical isolates in logarithmic phase at 37°C for 1 h. **(B)** Antibacterial activity of PlyA (100 μg/ml) against *P. aeruginosa* clinical isolates in logarithmic phase at 37°C for 1 h.

demonstrate that PlyA has broad antibacterial activity against MDR *A. baumannii* and *P. aeruginosa* isolates in Tris-HCl buffer.

Bacterial Phase Affects the Antibacterial Activity of PlyA

Although PlyA showed good antibacterial activity (from 6.8 log to 1.8 log) against logarithmic phase *A. baumannii* (**Figure 1C**), only a minor activity (from 6.8 log to 6.4 log) could be observed after treatment of the stationary phase *A. baumannii* WHG3066 cells with 50 μg/ml PlyA for 1 h (**Figure 3A**). Different susceptibilities to PlyA were also observed for logarithmic and stationary phase *P. aeruginosa* WHG3012 cells, but the stationary cells were still killed with a reduction of approximately 2 logs in cell number (**Figure 3B**). These results show that stationary phase *A. baumannii* and *P. aeruginosa* cells are not or partially killed in comparison to their logarithmic phase ones, respectively.

OMPs Enhance the Antibacterial Activity of PlyA Against Stationary Phase Cells

Outer membrane permeabilizers (OMPs), such as EDTA and citric acid have been used to enhance the bacteriolytic activity of lysins (Oliveira et al., 2014). However, an unneglectable minor

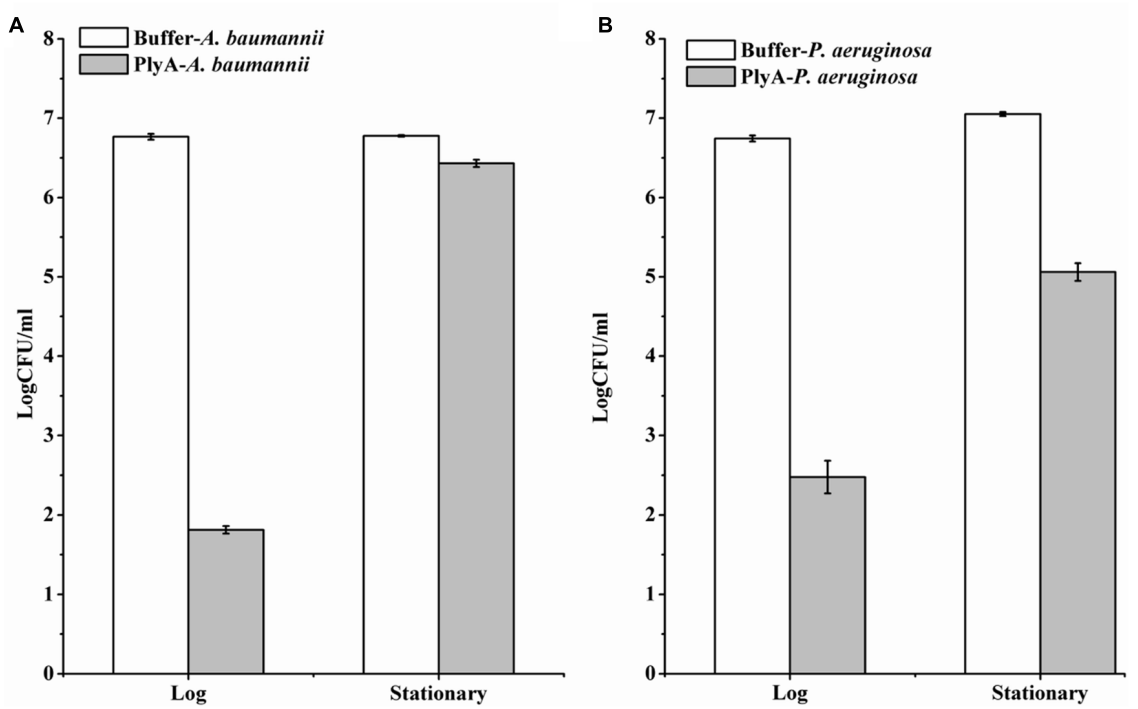

FIGURE 3 | Comparison between the activity of PlyA against *A. baumannii* WHG3066 (A) and *P. aeruginosa* WHG3012 (B) cells in logarithmic and stationary phase at 37°C for 1 h in 20 mM Tris-HCl (pH 7.4). Concentration of PlyA: 50 μg/ml.

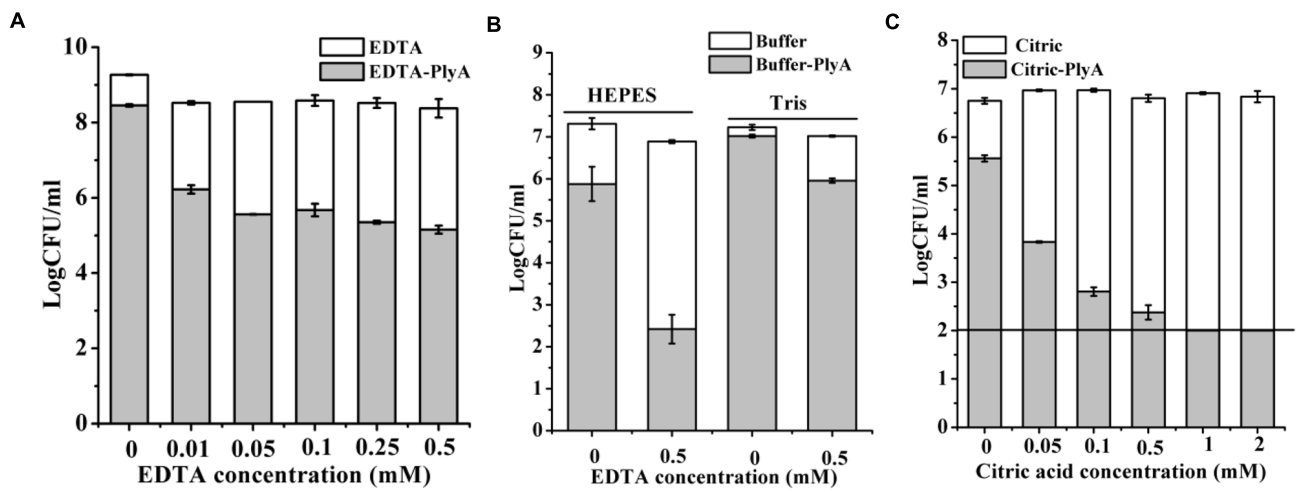

FIGURE 4 | Effects of EDTA and citric acid on the antibacterial activity of PlyA. (A,B) Effects of 100 μg/ml PlyA combined with various concentrations of EDTA against stationary phase *A. baumannii* WHG3066 in 20 mM Tris-HCl (pH 7.4) **(A)**, and *P. aeruginosa* WHG3012 **(B)** cells in different buffers at 37°C for 1 h. **(C)** Effects of 100 μg/ml PlyA combined with various concentrations of citric acid against stationary phase *A. baumannii* WHG3066 in 20 mM Tris-HCl (pH 7.4) with pH adjustment.

killing effect on logarithmic *P. aeruginosa* was noted in the presence of EDTA (Walmagh et al., 2012) and citric acid alone (Oliveira et al., 2014) previously by other researchers. Therefore, we only tested the synergism of these OMPs with PlyA against stationary phase cells. **Figure 4A** showed that EDTA alone has a minor killing effect on stationary phase *A. baumannii* cells, with a reduction of only 0.3 logs in 20 mM Tris-HCl (pH 7.4). Whilst, an obvious enhanced antibacterial activity of PlyA was observed in the presence of EDTA, with a reduction of 2.3–3.2 logs in the viable cell number. The synergism between EDTA and PlyA was observed not only in 20 mM Tris-HCl (pH 7.4), but also in 5 mM HEPES-NaOH (pH 7.4) against stationary

phase *P. aeruginosa* (with a reduction of 4.4 logs; **Figure 4B**). Because the acidification effect of citric acid was reported to kill logarithmic cells (Oliveira et al., 2014; Briers and Lavigne, 2015), we tested the effect of citric acid (0.05–2 mM) on PlyA antibacterial activity against the stationary phase cultures of *A. baumannii* by adjusting the pH values of the mixtures to 7.4 after adding citric acid to the Tris-HCl buffer. Results showed that a citric acid dose-dependent synergism between citric acid and PlyA was observed (**Figure 4C**), indicating that mechanisms other than the acidification effect exist underlying the synergism between citric acid and PlyA against the stationary phase cells.

Antibacterial Activity of PlyA in Bio-Matrix Conditions

Finally, we evaluated the performance of PlyA in some bio-matrix conditions, including LB culture medium, pasteurized milk, and human serum, which are more complicate than the pure Tris-HCl or HEPES-NaOH buffer. **Figure 5** showed that no antibacterial activity was observed in the presence of PlyA in these conditions against logarithmic phase *A. baumannii* cells. The abolished activity was also observed in MH, BHI, and TSBN culture media (data not shown). These results indicate that PlyA alone is easily inactivated in complicate environments, limiting its application only to relatively simple or special conditions.

DISCUSSION

The global emergence of MDR bacteria is calling to find novel molecules for treating the infections caused by them. Some recent studies on peptide-modified lysins or prophage lysins with similar structural compositions have shown that they could kill Gram-negative pathogens, including *P. aeruginosa* (Briers et al., 2014a) and *A. baumannii* (Lood et al., 2015). However, less is known about the conditions that influence the activities of peptide-modified lysins and their potential limitations in real applications. In the present study, we found that the activity of a new peptide-modified lysin could be affected by the bacterial growth phase and the bio-matrix, which should be taken into consideration for the development of new peptide-modified lysins.

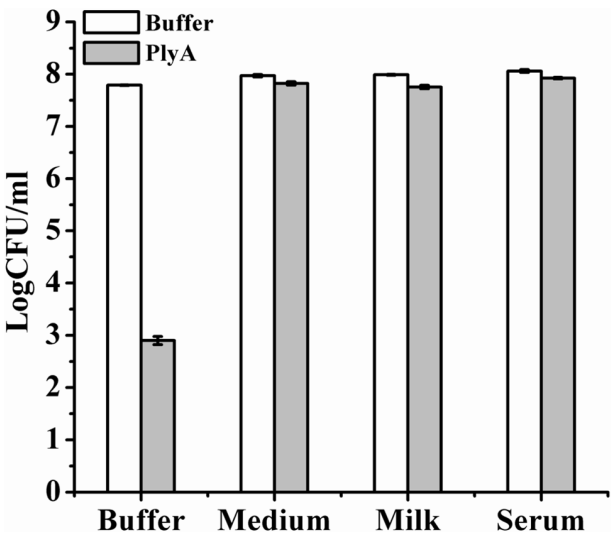

FIGURE 5 | Activity of PlyA in some matrices. Logarithmic phase *A. baumannii* WHG3066 cells were suspended in Tris-HCl buffer (pH 7.4), LB culture medium (pH 7.0), pasteurized milk (pH 6.8), and serum (pH 6.9), respectively. After treatment with 100 μg/ml PlyA at 37°C for 1 h, the viable cell numbers were calculated by plating on LA plates. Buffer treated groups were used as controls.

We observed that PlyA showed good antibacterial activity in Tris-HCl buffer against logarithmic phase *A. baumannii* and *P. aeruginosa* clinical isolates (**Figure 2**), including these with various antibiotic resistance profiles (**Table 1**, some of them are MDR isolates). These results demonstrate that the strategy of modifying lysins with a selected peptide is powerful to obtain novel engineered lysins against Gram-negative bacteria, including MDR isolates. The action model of a well known peptide-modified lysin, Artilysin®, is speculated to be: (1) the peptide fused in the N- or C-terminal of a target lysin interacts with the lipopolysaccharide of the Gram-negative bacteria, resulting in the destabilization and deformation of the outer membrane; (2) the lysin moiety transfers through the outer membrane driven by the self-promoted uptake of the peptide, and (3) thus gets access to and hydrolyze its peptidoglycan substrates, ultimately gives rise to cell lysis. This hypothesized mode of action was recently confirmed by Briers and coworkers in the time-lapse microscopy of *P. aeruginosa* cells exposed to Artilysin® LoGT-008 (Briers et al., 2014b). The TEM analysis of *A. baumannii* cells in this study also supports this hypothesis. As shown in **Figure 1**, PlyA disintegrates the cell wall of *A. baumannii*, and causes the loss of cytoplasmic contents in a single or multiple sites. This observation is quite similar with the typical phenomenon of osmotic-mediated cell lysis following the actions of phage lysins against Gram-positive bacteria reported elsewhere (Daniel et al., 2010).

Although PlyA showed good antibacterial activity against cells in logarithmic phase, a nearly abolished antibacterial activity was observed against stationary phase *A. baumannii* cells (**Figure 3A**). In case of stationary phase *P. aeruginosa* the antibacterial activity is not abolished but greatly reduced (**Figure 3B**). This observation is quite consistent with a recent report showing that a higher antibacterial activity of PlyF307 lysin was noted against exponentially growing *A. baumannii* (Lood et al., 2015). The different susceptibility may be due to the different structure and composition of the cell membranes between logarithmic and stationary phase cells. The outer membrane of bacteria is mainly composed of lipopolysaccharides, phospholipids and proteins, and their contents and types are varied in different environmental conditions and growth phases (Cronan, 1978). Additionally, there may be also difference in peptidoglycan architecture between logarithmic and stationary phase cells, such as the thickness of peptidoglycan layer, which influences their susceptibility to PlyA.

The strong synergisms between PlyA and OMPs (EDTA and citric acid in Tris-HCl and HEPES-NaOH buffer) against stationary phase cultures of *A. baumannii* (**Figures 4A,C**) and *P. aeruginosa* cells (**Figure 4B**) indicate that the outer membrane of Gram-negative bacteria is indeed a physical barrier for the bacteriolytic activity of natural lysins. By combining with OMPs, PlyA may be helpful in *ex vivo* and topical applications, such as environmental or surface disinfection, but not suitable for systemic infections due to the potential risk of anti-coagulating properties of the OMPs.

Other researchers have noted that protonated form of citric acid has both chelating effect and acidification effect on bacterial cells, and the acidification effect could damage the outer membrane of Gram-negative bacteria (such as *P. aeruginosa*) and cause a reduction in viable cell number (Oliveira et al., 2014; Briers and Lavigne, 2015). However, in the present study, we found that the acidification-killing effect of citric acid (2 mM) was only observed in logarithmic cultures of *A. baumannii*, but not stationary phase ones (data not shown). Moreover, an obvious synergism between citric acid and PlyA was observed against the stationary phase cultures of *A. baumannii* in a citric acid dose-dependent manner when pH values were adjusted to neutral pH (**Figure 4C**). These results indicate that it is the chelating effect, but not acidification effect of citric acid, which enhanced the antibacterial activity of PlyA against stationary phase cultures of *A. baumannii*.

One serious shortcoming for PlyA may be its inactivation in complicate bio-matrices, including media, milk and serum (**Figure 5**), which means PlyA could only be used in relatively simple or specified conditions, such as material and skin surface disinfection. One possible reason for the inactivation of PlyA may be the conjugation and passivation of negatively charged molecules present in these matrices to the positively charged peptides, which may render the peptide losing the outer membrane penetrating activity.

However, in the previous reports, the efficacies of some Artilysin® s have been demonstrated in *P. aeruginosa* infected *Caenorhabditis elegans* model and human keratinocytes monolayer model in the presence of EDTA (Briers et al., 2014b), as well as in *in vitro* case studies of dog otitis caused by *P. aeruginosa* (Briers and Lavigne, 2015). Therefore, it seems that it is not easy and straight-forward to design an engineered peptide-modified lysin which shows robust activity under infection conditions as described for Artilysin® s. Some optimization like fusing different peptides with different endolysins and combining with different linkers is required to obtain an antibacterial lysin with the desired properties and a robust activity (Briers et al., 2014b).

CONCLUSION

We report here a newly engineered lysin, PlyA, with high bacteriolytic activity against *A. baumannii* and *P. aeruginosa in vitro*. This study also indicated that conditions such as bacterial growth phase and the bio-matrix can influence the antibacterial activity of PlyA, suggesting that there are still some limitations that should be taken into consideration for the development of new peptide-modified lysins, and optimization is needed to obtain an antibacterial lysin with robust antibacterial activity.

AUTHOR CONTRIBUTIONS

HY and HW conceived the study. HY and MW performed experiments. HY, JY, and HW analyzed data. HY and HW wrote the paper.

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frontiers in Microbiology

ORIGINAL RESEARCH
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Structural and Enzymatic Characterization of ABgp46, a Novel Phage Endolysin with Broad Anti-Gram-Negative Bacterial Activity

Hugo Oliveira[1], Diana Vilas Boas[1], Stéphane Mesnage[2], Leon D. Kluskens[1], Rob Lavigne[3], Sanna Sillankorva[1], Francesco Secundo[4] and Joana Azeredo[1]**

[1] Centre of Biological Engineering, Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, Braga, Portugal, [2] Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, UK, [3] Laboratory of Gene Technology, KU Leuven, Leuven, Belgium, [4] Istituto di Chimica del Riconoscimento Molecolare – Consiglio Nazionale delle Ricerche, Milano, Italy

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Rustam Aminov,
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Tadeusz Kaczorowski,
University of Gdańsk, Poland

***Correspondence:**
Joana Azeredo
jazeredo@deb.uminho.pt;
Hugo Oliveira
hugooliveira@deb.uminho.pt

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The present study demonstrates the antibacterial potential of a phage endolysin against Gram-negative pathogens, particularly against multidrug resistant strains of *Acinetobacter baumannii*. We have cloned, heterologously expressed and characterized a novel endolysin (ABgp46) from *Acinetobacter* phage vb_AbaP_CEB1 and tested its antibacterial activity against several multidrug-resistant *A. baumannii* strains. LC-MS revealed that ABgp46 is an *N*-acetylmuramidase, that is also active over a broad pH range (4.0–10.0) and temperatures up to 50°C. Interestingly, ABgp46 has intrinsic and specific anti-*A. baumannii* activity, reducing multidrug resistant strains by up to 2 logs within 2 h. By combining ABgp46 with several organic acids that act as outer membrane permeabilizing agents, it is possible to increase and broaden antibacterial activity to include other Gram-negative bacterial pathogens. In the presence of citric and malic acid, ABgp46 reduces *A. baumannii* below the detection limit (>5 log) and more than 4 logs *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. Overall, this globular endolysin exhibits a broad and high activity against Gram-negative pathogens, that can be enhanced in presence of citric and malic acid, and be used in human and veterinary medicine.

Keywords: *Acinetobacter baumannii*, phage endolysins, mass spectrometry, circular dichroism, antibacterial activity

INTRODUCTION

Gram-negative (G^-) pathogenic bacteria remain a global human health concern as they are common causes of foodborne, environmentally acquired, and zoonotic infectious diseases (Chopra et al., 2008; Scallan et al., 2011). This problem is exacerbated by their ability to display intrinsic [e.g., low outer membrane (OM) permeability] or acquired antibiotic resistant mechanisms (e.g., horizontal transfer of resistance genes; Bonomo and Szabo, 2006). *Acinetobacter baumannii* and *Pseudomonas aeruginosa* cause a wide range of nosocomial diseases (including wound, skin infections) and are characteristic strains for which resistance has occurred against and spread to nearly all antibiotics available. The development and spread of multidrug resistant pathogens have instigated and intensified the interest in alternative antimicrobials.

Bacteriophage (phages) lytic enzymes, called endolysins, are proteins synthesized at the end of the phage lytic cycle to destroy the cell wall peptidoglycan (PG) to release viral progeny

(Fischetti, 2005). Endolysins have either a single catalytic domain (globular structure) responsible for the PG digestion by glycosidase, amidase or peptidase activity, typically observed in phages infecting G^- hosts, but can contain a second domain [modular structure, an architecture more prominent in Gram-positive (G^+)-like endolysins] that aids substrate recognition and binding (Oliveira et al., 2012). Recent *in silico* analysis illustrated the large diversity and complexity of endolysins that can comprise up to four domains in the same coding sequence, have 24 different types of catalytic and 15 binding domains, and 89 possible architectural organizations (Oliveira et al., 2013). In view of this tremendous diversity, endolysins have received relatively little attention, especially in terms of more fundamental aspects such as their conformation analysis as well as their its anti-G^- activity.

Due to their biotechnological potential of endolysins including their unique ability to rapidly cleave the bacterial PG through external application and the absence of reported resistance development, endolysins have sparked the interest as alternatives for existing antibiotics (Nelson et al., 2001). However, a review of the literature still demonstrates an overwhelming and almost exclusive application of endolysins in combating G^+ pathogens (Fischetti, 2010). This has been shown, for example, on methicillin-resistant and multidrug-resistant *Staphylococcus aureus* (endolysins LysK and MV-L, respectively; O'Flaherty et al., 2005; Rashel et al., 2007), on vancomycin-resistant *Enterococcus faecalis* and *E. faecium* (endolysin PlyV12; Yoong et al., 2004). The limited number of publications against G^- bacteria is explained by permeabilization issues due to the presence of the OM that hinders the action of externally added endolysins (Vaara, 1999). To date, only a few endolysins were successfully reported to kill G^- cells (Lood et al., 2015), mostly combining endolysin activity with the chelating effect of EDTA to permeabilize the OM and, more recently, by novel engineering strategies, for specific applications (Lim et al., 2012; Walmagh et al., 2012; Briers et al., 2014a,b; Lood et al., 2015). Recently, the application of endolysins in combination with weak acids (citric and malic acid), also proved to be successful in eliminating a broad range of G^- pathogens (Oliveira et al., 2014).

This study describes the isolation of a phage-encoded endolysin (ABgp46) originating from an *Acinetobacter* phage vb_AbaP_CEB1. ABgp46 was fully characterized in terms of its primary, secondary and tertiary structure under different physiological conditions giving novel insights into this class of proteins. Its antibacterial action was studied by combining the endolysin with several organic acids (citric, malic, lactic, benzoic, and acetic acid) against antibiotic resistant G^- bacteria. This work demonstrates that weak acids are suitable OM permeabilizers (OMPs), enabling ABgp46 to reach the PG and kill the cells.

MATERIALS AND METHODS

Bacterial Strains, Media, and Chemicals

Bacterial strains were acquired either from the American Type Culture Collection (*S. typhimurium* ATCC 19585, *P. aeruginosa* ATCC 15692, and *Klebsiella oxytoca* ATCC 131821) or from the Spanish Type Culture Collection (*Escherichia coli* O157:H7 CECT 47821). Clinical *Acinetobacter* isolates are multi-resistant and were kindly provided by the Hospital of Braga with patterns of antibiotic resistance given for each strain. All strains were grown in Lysogeny Broth (LB; Liofilchem) at 37°C and 120 rpm. For transformation, chemically competent *E. coli* TOP10 and *E. coli* BL21(DE3) cells (Invitrogen) were prepared for cloning and recombinant protein expression respectively. The *Acinetobacter* phage vb_AbaP_CEB1 (encoding the ABgp46 endolysin) was isolated from waste effluents and belongs to the Centre of Biological Engineering phage collection (Braga, Portugal). This phage belongs to the *Podoviridae* family and is a T7 likeviruses EDTA was acquired from Pronalab while isopropyl-β-D-thiogalactopyranoside (IPTG), HEPES and the organic acids were purchased from Sigma-Aldrich.

Cloning, Recombinant Protein Expression, and Purification

Phage DNA was extracted from concentrated purified phage lysates using the phenol:chloroform extraction method (Sambrook et al., 2001). Afterward, the ABgp46 ORF was amplified from the phage genomic DNA using Phusion High-Fidelity DNA Polymerase (NEB) and a set of specific primers (forward: 5-GGCAGCCATATGATTCTGACTAAAGACGGGT TTAG and reverse: 5-GCAGCCGGATCCCTATAAGCTCCGTA GAGCGC, with the NdeI/BamHI restriction endonuclease sites underlined). Next, the amplicon was purified (DNA Clean & Concentrator-5k, Zymo Research), digested using NdeI and BamHI enzymes (NEB), and cloned in the pET15b expression vector (Novagen) with an N-terminal His6-tag. The insert was confirmed by Sanger sequencing (Eurofins). The sequence of ABgp46 was deposited in GenBank under accession no. KP998152.

Escherichia coli BL21(DE3) was transformed with the pET15b-ABgp46 vector and the endolysin produced as follows: cells were grown in 200 mL LB (supplemented with 100 μg/mL of ampicillin) to an $OD_{600\ nm}$ of 0.6 (approximately 4 h, 120 rpm at 37°C; ES-20/60), and recombinant protein expression induced with 0.5 mM IPTG at 16°C, 120 rpm overnight. The culture was then centrifuged (9500 × *g*, 30 min), and the cells disrupted by suspending the pellet in 1/25 volumes of lysis buffer (20 mM NaH_2PO_4, 0.5 M NaCl/NaOH, pH 7.4), followed by three cycles of freeze-thawing (−80°C to room temperature). Maintaining the sample on ice, cells were further disrupted by sonication (Cole-Parmer, Ultrasonic Processors) for 8–10 cycles (30 s pulse, 30 s pause). Insoluble cell debris was removed by centrifugation (9500 × *g*, 30 min, 4°C) and the supernatant was collected, filtered (0.22 μm filters, GE Healthcare) and applied to Ni^{2+}-NTA resin stacked in HisTrapTM HP 1 mL columns (GE Healthcare) for purification, using a imidazole gradient (25–300 mM). Eluted protein fractions were visualized by standard denaturation SDS-PAGE gels, and only protein fractions with purity >95% were dialyzed in 10 mM PBS at pH 7.2 (using Maxi GeBAflex-tube Dialysis Kit – Gene Bio-Application Ltd). The protein was quantified using the BCA TM Protein Assay Kit (Thermo Scientific).

Endolysin Activity Characterization

Detection and Quantification of Muralytic Activity

The assessment of the ABgp46 muralytic (PG degrading) activity was performed by visualizing inhibition spots on G^- bacteria lawns, as described elsewhere (Schuch et al., 2009). Briefly, 100 μL of *Acinetobacter* strains grown to an $OD_{600\ nm}$ of 0.6 were spread over the surface of LB agar and grown overnight at 37°C. Formed bacterial lawns were subsequently treated with chloroform vapors for 30 min to induce OM permeabilization, after which a 30-μL drop of purified ABgp46 was spotted. After a few minutes, the visualization of inhibition halos determined the presence of a endolysin activity, for its ability to degrade the exposed PG.

The muralytic activity of ABgp46 was quantified on permeabilized *P. aeruginosa* cells following treatment with chloroform/Tris-HCl to allow the protein to reach its PG substrate (Lavigne et al., 2004). Briefly, mid-exponential *P. aeruginosa* strains were centrifuged (4600 × *g*, 30 min at 4°C), suspended in the upper layer of chloroform-saturated 50 mM Tris-HCl, pH 7.7 solution and incubated for 45 min at 120 rpm. Following incubation, bacterial protoplasts were collected (4000 × *g*, 15 min, 4°C), washed and suspended in 80 mM of KH_2PO_4/K_2HPO_4 buffer (pH 7.2) then adjusted to an $OD_{600\ nm}$ of 1.2. Bacterial protoplasts were stored at −20°C prior to use. To measure enzymatic activity, 30 μL of enzyme (0.125, 0.25, 0.5, 1, 2, 4, and 8 μM) were added to 270 μL of permeabilized *P. aeruginosa* cells. The resulting decrease in optical density was measured spectrophotometrically (BIO-TEK®, Synergy HT Microplate Reader) for up to 30 min of reaction with readings taken every 30 s. To measure the pH dependence, chloroform/Tris-HCl permeabilized OM *P. aeruginosa* cells were suspended in a universal pH buffer (10 mM KH_2PO_4, 10 mM Na-citrate and 10 mM H_3BO_4), with a pH adjusted between 4.0 and 10.0. Obtained OD values were adjusted for the contribution of the negative control (PBS treated). The muralytic activity was calculated based on the best linear regression of the saturation curve and expressed in units/μM according to a validated method explained in detail elsewhere (Briers et al., 2007).

Stability

The enzyme stability was tested using similar turbidity tests as described previously (Oliveira et al., 2014). Enzyme kinetics experiments were performed using 2 μM of ABgp46 stored at 4°C for 2 months, or heated at different temperatures of 20, 30, 40, 50, and 60°C for 30 min in a MJ Mini BIO-RAD Thermocycler, in the universal buffer at optimal pH. The residual muralytic activity of each sample relative to the activity of the unheated reference sample at time 0 (=100% activity) was determined.

Determination of the PG Cleavage Site

The ABgp46 cleavage site was determined using *E. coli* BW25113 Δ*lpp* PG sacculi as a substrate. PG was extracted from exponentially growing *E. coli* cells as previously described using boiling SDS (Glauner, 1988). Five hundred μg of pure PG was digested overnight with 0.6 mg/ml of ABgp46 at 37°C in 25 mM of phosphate buffer pH 6.0, supplemented with 0.1 mM of $MgCl_2$ in a final volume of 100 μl. *E. faecalis* AtlA *N*-acetylglucosaminidase (0.6 mg/ml) and *S. globisporus N*-acetylmuramidase mutanolysin (Sigma-Aldrich; 500 μg/ml) were used as controls to digest 500 μg of *E. coli* PG in 25 mM Tris-HCl (pH 8.0) and 25 mM of phosphate buffer (pH 6.0), respectively. Soluble muropeptides were recovered by centrifugation (20,000 × *g*, 15 min, 20°C), reduced with 5 mg/ml of sodium borohydride and separated by reverse-phase HPLC (RP-HPLC) on a Hypersil aQ C_{18} column (3 μm; 2.1 by 200 mm; ThermoFisher Scientific) connected to an Agilent 6500 Series Q-TOF LC/MS System. Muropeptides were eluted at a flow rate of 0.25 ml/min with a 0 to 15% gradient applied between 6 and 40 min [Buffer A, 0.1% (v/v) formic acid in water; buffer B, 0.1% (v/v) formic acid in acetonitrile].

Endolysin Conformation Stability

Fluorescence measurements

The intrinsic fluorescence emission spectrum of the ABgp46 was measured over the 300–400 nm range using a Jasco FP-750 spectrofluorimeter equipped with a Peltier thermostat. An excitation wavelength of 295 nm was used to minimize the emission arising from tyrosine residues. Thermal denaturation was monitored by heating 2 μM protein in universal buffer with a constant rate of 2°C/min from 20 to 70°C. The variation of the fluorescence spectra was measured as a variation of Parameter A defined as the ratio of intensity of fluorescence (IF) at 360 and 325 nm (IF 360/IF 325). Parameter A was plotted as a function of temperature and fitted in Boltzmann sigmoidal curves.

Circular dichroism analysis

Endolysin circular dichroism (CD) spectra were recorded in triplicate in the Far-UV region (195–260 nm) using a J-1100 CD Spectrometer, Jasco, in the universal buffer (pH 4.0–10.0). Spectra were recorded at desired pH, from 20 to 70°C, with a thermal increase rate of 2°C/min. All CD spectra were baseline corrected and smoothed with the Spectra Analysis JASCO software. The intensity of the CD signal measured at 222 nm was plotted as a function of temperature and fitted in Boltzmann sigmoidal curves. In all cases, an enzyme concentration of 8 μM (0.18 mg/mL) and an optical path of 0.1 cm were used.

Antibacterial Assays

In vitro assays on planktonic cells were performed as described previously (Oliveira et al., 2014), with minor modifications. Mid-exponential phase cells ($OD_{600\ nm}$ of 0.6) suspended and 100-fold diluted in 10 mM HEPES/NaOH (pH 7.2), were prepared. Each culture (50 μL) was incubated for 2 h at room temperature with 25 μL of ABgp46 (final concentration of 2 μM) together with 25 μL of water or 25 μL or OMPs (EDTA, citric, malic, lactic, benzoic, or acetic acid) dissolved in water. Parallel experiments were also carried out supplementing the reaction with 5 mM of $MgCl_2$ to evaluate the effect of free divalent cations in the solution. In all cases, negative controls were included by incubating 50 μL of cells with 25 μL of PBS (pH 7.2; replacing ABgp46) or with 25 μL

water (instead of OMPs). After incubation, CFUs were counted in LB agar plates and the antibacterial activity quantified as the relative inactivation in logarithmic units [$= \log_{10}(N_0/N_i)$ with N_0 = number of untreated cells (in the negative control) and N_i = number of treated cells counted after incubation]. Averages ± standard deviations for all experiments are given for $n = 4$ repeats.

RESULTS

In silico Analysis of the Primary Structure

Acinetobacter phage vB_AbaP_CEB1 was previously isolated and sequenced (unpublished data). ORF 46 (referred to as ABgp46), encoding a 185-amino acid protein with a deduced molecular mass of 23.1 kDa, is predicted to act as a PG hydrolase with a HHpred output showing that ABgp46 belongs to the CAZY glycosidase family 19 (GH19; E-value $= 2 \times 10^{-36}$; Cantarel et al., 2009). GH19 represents a class of chitinases that cleaves the unbranched chains of *N*-acetyl glucosamine polymers, a structure uncommon in bacterial cell walls, but some enzymes that able to degrade the PG of G^- bacteria have also been shown (e.g., *Pseudomonas* phage OBP and *Salmonella* phage PVP-SE1 endolysins; Walmagh et al., 2012).

BlastP analyses showed that the protein has a high sequence similarity to four other predicted endolysins from *Acinetobacter* phages (phiAB1, phiAB3, ABP-01, and ABP-04). Amphipathic helices were identified between the amino acids 112 and 145 (**K**NPE**K**ALEPLIAIQIAI**K**GMLNGWFTGVGF**RRKR**), with positively charged amino acids shown in bold. The same sequence has been observed in the *A. baumannii* phage endolysin LysAB2, which has been shown to interfere with the *A. baumannii* OM (Lai et al., 2011). ABgp46 was produced as a recombinant protein in *E. coli* and purified under native conditions, yielding a soluble protein of 20.5 mg per liter of culture.

Endolysin Muralytic Activity, pH Dependence, and Stability

ABgp46 was able to lyse OM compromised *A. baumannii* #2 lawn(phage host) and its activity calculated using turbidimetry assays. ABgp46 is active between of pH of 4.0–9.0 with an optimal between 8.0 and 9.0 (**Figure 1A**). At optimal pH, ABgp46 had a muralytic activity of 490 units/μM. In addition, ABgp46 remained fully active after 1 month at 4°C with a 25% decrease in activity observed after 30 min incubation at 50°C and complete inactivation after 30 min incubation at 60°C (**Figure 1B**).

RP-HPLC and LC-MS Analysis of the Biochemical Activity of ABgp46

The PG bond cleaved by ABgp46 was identified by LC-MS (Supplementary Figures S1 and S2) using *E. coli* purified PG as a substrate. The muropeptide profiles corresponding to the digestion of *E. coli* PG by ABgp46, *E. faecalis* AtlA (an *N*-acetylglucosaminidase) and mutanolysin (an *N*-acetylmuramidase) were similar, suggesting that ABgp46 displays glycosyl hydrolase activity. MS analysis of the muropeptides corresponding to the major monomers solubilized (peaks 1–3 in Supplementary Figure S1) identified ions with *m/z* values of 942.415, 942.414, and 942.414, respectively, matching the theoretical value expected for a disacharride-tetrapeptide (942.414). To identify whether ABgp46 is an *N*-acetylmuramidase or an *N*-acetylglucosaminidase, we analyzed the fragmentation pattern of the ions with an *m/z* at 942.414 to identify the sugar moiety harboring a reducing group (Eckert et al., 2006). Major ions in peaks 1 (mutanolysin digestion) and 3 (ABgp46 digestion) both revealed a fragmentation event leading to the loss of a non-reduced GlcNAc residue (−203.078 atomic mass units, seen as a $[M+H]^+$ adduct with *m/z* at 204.086 and 204.087) indicating that ABgp46 displays *N*-acetylmuramidase (lysozyme) activity. By contrast, the major ion in peak 2 (AtlA digestion) revealed a

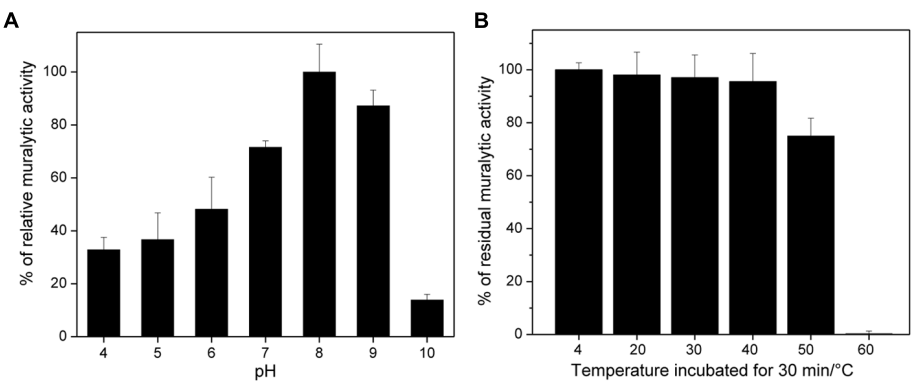

FIGURE 1 | pH and temperature effect on the ABgp46 activity. (A) The pH dependence was measured as the slope of the $OD_{600\ nm}$/min curve, using *Pseudomonas aeruginosa* OM permeabilized cells as a substrate, suspended in a universal buffer (10 mM KH_2PO_4, 10 mM Na-citrate, and 10 mM H_3BO_4), and with pH adjusted from 4.0 to 10.0. **(B)** The stability was tested by heating the protein at different temperatures (20–60°C) for 30 min, and using OM permeabilized *P. aeruginosa* PAO1 cells at pH of 8.0. The residual activity shown, is expressed in percentage by comparing with ABgp46 stored at 4°C (=100% relative activity). Averages and standard deviations of four repeated experiments are shown.

fragmentation event leading to the loss a reduced GlcNAc residue (−223.106 atomic mass units, seen as a $[M+H]^+$ adduct with an *m/z* at 224.113) expected for an *N*-acetylglucosaminidase activity (Supplementary Figure S2). Collectively, these results demonstrate that ABgp46 displays *N*-acetylmuramidase activity.

Conformational Analysis of ABgp46

An analysis of the ABgp46 tertiary and secondary structure was performed under different pH values, using CD and protein intrinsic fluorescence, respectively. The fluorescence spectra of ABgp46 obtained at different pH values are depicted in **Figure 2A**. Using the presence of the two tryptophan residues (Trp-95 and Trp-135) in the sequence, an excitation wavelength of 295 nm was used, minimizing the contribution of tyrosine fluorescence. In general, when increasing the temperature from 20 to 70°C, Parameter A showed a similar trend for all pH value and a marked variation was observed at 50°C. Exceptions were pH 4.0 and 10.0 with a Parameter A variation at lower temperatures in the range of 45–46°C. Such spectral variations can be correlated to folding changes of the tertiary structure.

Circular dichroism measurements revealed signal minima around 208 and at 220 nm that prevail in the far-UV CD spectra, which is indicative of α-helices in this protein. The same CD spectra were obtained as a function of pH adjusted from 4.0 to 10.0, indicating an unchanged protein secondary structure (data not shown). To determine the enzyme's conformational stability in depth, a thermal treatment was employed by increasing temperature of the protein solution at different pH values (**Figure 2B**). Secondary structure changes were analyzed by monitoring CD intensity at 222 nm where α-helix structures show a negative peak. No differences were observed between pH 5.0–9.0, where all T_m were recorded in the range of 51–52°C. Extreme pH values of 4.0 and 10.0 were less favorable conditions to maintain the secondary structure integrity, with T_m of 44.7 and 44.4°C, respectively. Interestingly, the pH 4.0 was the only condition where the protein did not aggregate after reaching the T_m, i.e., thermal unfolding. Overall, based on the Parameter A and the ellipticity data, it is likely that tertiary and secondary structure starts to melt at 40°C, resulting in unfolded and denatured protein above 50°C.

In vitro Antibacterial Activity

The *in vitro* antibacterial activity of the recombinant ABgp46 was investigated on a wide set of G^- strains (**Table 1**). The activity of Abgp46 alone on tested cells was insignificant as expected, with the exception of *A. baumannii* strains. Interestingly, some *Acinetobacter* strains were mildly sensitive to the endolysin alone, causing between 1 and 2 log reductions of viable cells, including strains resistant to several antibiotics.

To broaden and increase the antimicrobial effect of the Abgp46 to other G^- pathogens, *E. coli* O157:H7 CECT 4782 was chosen as a model strain, to test and optimize an ABgp46/OMP formula. Several organic acids (citric and malic, lactic, benzoic, and acetic acid) were used to sensitize the G^- to the endolysin by permeabilizing the OM. Each OMP was used in different concentrations (to achieve three distinct reaction pH values of 7.0, 5.5, and 4.0) and combined with ABgp46 to assess the best antibacterial condition after 2 h incubation (illustrated in **Table 2**). At pH 7.0, only the ABgp46/citric acid was able to slightly reduce *E. coli* cells (0.86 ± 0.28 logs; **Table 2**). At pH 5.5, the effect was broadened to ABgp46/citric acid, ABgp46/malic acid and ABgp46/lactic acid mixtures reducing approximately 1 log of *E. coli* cells (**Table 2**). Consistently, the effect was more pronounced at pH of 4.0. At this pH, the combinatorial effects of ABgp46/citric acid, ABgp46/malic acid, and ABgp46/lactic acid mixtures resulted in 2.78 ± 0.37, 2.08 ± 0.32, and 0.94 ± 0.28 log reductions, respectively (**Table 2**). In this condition, the antibacterial effect was also extended to ABgp46/benzoic acid inactivating approximately 1 log of *E. coli* cells. No effect was observed with ABgp46/acetic acid. Taking into account

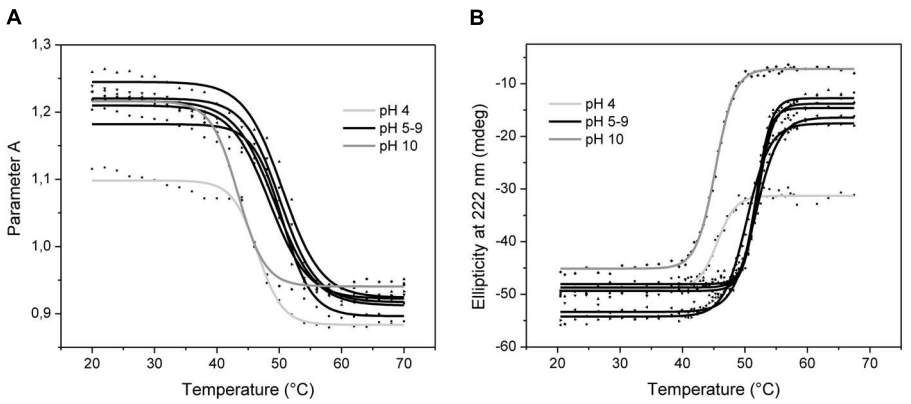

FIGURE 2 | Variation of ABgp46 tertiary and secondary structure with pH and temperature. (A) Correlation between fluorescence spectra and conformational changes deduced by plotting Parameter A as a function of the same pH and temperature ranges, for tertiary structure analysis. **(B)** Far-UV Circular dichroism thermal denaturation profiles (from 20 to 70°C, with heating rates of 2°C/min), measured at 222 nm, and at different pH values (pH 4.0–10.0) for secondary structure analysis.

the most efficient conditions tested, ABgp46/OMPs mixtures (OMPs concentrations used to achieve a pH of 4.0) were tested in the presence of 5 mM $MgCl_2$. $MgCl_2$ provides an extra source of available divalent cations (Mg^{2+}) that can link to the LPS negatively charged phosphate groups, strengthening the LPS monolayer (Vaara, 1999). Therefore, the addition of $MgCl_2$ completely abolished the activity of all ABgp46/OMP combinations.

After optimization, citric (at 3.65 mM) and malic (at 4.55 mM) acids were selected as the best OMPs to be combined with ABgp46. Their synergistic effect was further tested on the remaining unsensitized G^- strains tested earlier and compared with the gold standard EDTA (at 0.5 mM) OMP (**Table 3**). In the presence of OMPs and ABgp46 no surviving *A. baumannii* cells of strain #2 (phage host strain), could be detected (>5 log reduction; limit of detection, <10 CFU/ml). In addition, *P. aeruginosa* and *S. typhimurium* showed a greater than 4 log reduction when combined with citric or malic acid. Lower, yet still significant reductions, ranging from 1 to 2 logs, were observed against *Cronobacter sakazakii*. In case of *K. oxytoca*, only the ABgp46/citric acid and ABgp46/malic acid combinations showed a moderate effect with log reductions of 0.96 ± 0.35 and 0.81 ± 0.12 viable cells, respectively.

Overall, an antibacterial effect was observed when ABgp46 was combined with different OMPs, and its effect was found to be more pronounced in the presence of citric or malic acid than when the chelating agent EDTA was used.

TABLE 1 | Antibacterial activity of ABgp46 against several Gram-negative bacterial pathogens.

Bacterial species	ABgp46/water	Origin/characteristics
P. aeruginosa PAOI	0.03 ± 0.02	Reference strain (ATCC 15692)
S. typhimurium LT2	0.14 ± 0.05	Clinical strain
E. coli 0157:H7	0.17 ± 0.12	Reference strain (CECT 4782)
C. sakazakii	0.23 ± 0.26	Reference strain (CECT 858)
K. oxytoca	0.18 ± 0.13	Reference strain (ATCC 13182)
A. baumannii #1*	**1.79 ± 0.38**	Clinical strain (TI, AMC, TZP, CE, AZ, ME, AN, GM, NN, CP, PF, TS)
A. baumannii #2*	**0.93 ± 0.25**	Clinical strain (TI, AMC, TZP, CE, AZ, ME, AN, GM, NN, CP, PF, TS)
A. baumannii #3*	**1.00 ± 0.32**	Clinical strain (TI, AMC, TZP, CE, CEF, AZ, ME, GM, NN, CP, PF, TS)
A. baumannii #4*	**0.81 ± 0.21**	Clinical strain (TI, AMC, TZP, CE, CEF, AZ, ME, GM, NN, CP, PF, TS)
A. baumannii #5*	0.37 ± 0.19	Clinical strain (TI, AMC, TZP, CE, CEF, AZ, ME, CP, PF)
A. baumannii #6*	**1.20 ± 0.46**	Clinical strain (TI, AMC, TZP, CE, CEF, AZ, ME, CP, PF)

*Averages ± standard deviations for all experiments are given for n = 4 repeats. Marked in bold are ≥1 log reduction units observed. *Known patterns of antibiotic resistance provided by the Hospital of Braga, based on susceptibility tests performed according to the NCCLS for antibiotic resistance; TI, Ticarcillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; CE, ceftazidime CEF, Cefepime; AZ, Aztreonam, ME, meropenem; AN, amikacin; GM, gentamicin; NN, tobramycin; CP, ciprofloxacin; PF, Pefloxacin; TS, Trimethoprim/Sulfametoxazol.*

DISCUSSION

G^- bacterial infections have always been a threat to human health. In particular, infections caused by antibiotic resistant bacteria are problematic and their incidence is constantly reported worldwide (Chopra et al., 2008; Scallan et al., 2011). Phage-encoded endolysins represent one promising avenue of investigation to fight these pathogens.

Biochemical characterization assays were carried out with ABgp46 to expand the knowledge of the G^--like endolysins activity and their conformational stability. LC-MS experiments demonstrated that ABgp46 is an *N*-acetylmuramidase, cleaving the PG between MurNAc and GlcNAc glycan strands. Compared to previously reported G^--like globular endolysins (Supplementary Table S1), ABgp46 has a similar muralytic activity, and is more active at alkaline conditions (pH 8.0–9.0; Lai et al., 2011; Walmagh et al., 2013). Like others (Supplementary Table S1), ABgp46 also retains its activity once refrigerated for at least 1 month, and only few endolysins are regarded as heat-resistant proteins (Walmagh et al., 2012, 2013). The biochemical characterisation of the endolysin was also complemented by analyzing the stability of its structure. In this study, we employed fluorescence and CD studies that indicated that ABgp46 has a melting temperature of 52°C, at the optimal pH value. The good correlation between muralytic activity and conformational changes as a function of temperature strongly suggests that the decrease of activity observed for ABgp46 above 50°C is mainly due to structural protein modifications. Similar studies have only been conducted with a *Salmonella* phage endolysin Lys68, with an observed melting temperature of 44°C (Oliveira et al., 2014).

To evaluate the potential use of ABgp46 as an antimicrobial compound, the enzyme was first tested without the addition of a permeabilizer. Interestingly, despite the present of the OM protecting the PG layer, externally added ABgp46 was active against several multi-resistant *Acinetobacter* strains (14 out of 17 antibiotics). This is a rare event reported for G^--like endolysins. The T4 lysozyme (from the T4 phage), was the first endolysin described to naturally kill G^- cells. It was shown that its C-terminal positively charged amphipathic α-helix (named α-4) is responsible to cause OM disruption, having a stronger bactericidal effect than the enzymatic PG hydrolysis (During et al., 1999). Later, four other endolysins (OBPgp279, Lys1521, PlyF307, and LysAB2) were also reported to spontaneously inactivate G^- cells (Morita et al., 2001; Lai et al., 2011; Walmagh et al., 2012; Lood et al., 2015). However, opposite to the bactericidal effect of synthetic α-4 of T4 lysozyme, it is assumed that the C-terminal positively charged region of these endolysins does not have an antibacterial effect *per se*, but rather mediate the N-terminal enzymatic domain to enter the cells, allowing them to digest the PG and cause bacteriolysis. In general, these endolysins capable of spontaneously killing G^- bacteria show a very specific antibacterial activity. This intrinsic antibacterial activity that was also observed for ABgp46 can be explained by the same C-terminal amphipathic region (amino acid sequence between 112 and 145) identified in LysAB2.

To improve the antibacterial activity, ABgp46 was combined with concentrations of OMP agents (citric, malic, lactic, benzoic,

TABLE 2 | Combinatorial antibacterial activity of ABgp46 with outer membrane permeabilizers under different concentrations against *Escherichia coli* O157:H7.

pH	OMPs	Molarity (mM)	PBS/OMPs	ABgp46/OMPs	ABgp46/OMPs + 5 mM $MgCl_2$
7.0 ± 0.1	EDTA	0.50	0.27 ± 0.11	0.32 ± 0.15	
	Citric	0.36	0.21 ± 0.14	**0.86 ± 0.28**	
	Malic	0.60	0.24 ± 0.11	0.53 ± 0.28	
	Lactic	1.20	0.24 ± 0.14	0.55 ± 0.31	
	Benzoic	1.20	0.26 ± 0.15	0.49 ± 0.34	
	Acetic	1.20	0.25 ± 0.14	0.53 ± 0.30	
5.5 ± 0.1	Citric	1.50	0.20 ± 0.13	**1.45 ± 0.25**	
	Malic	3.30	0.17 ± 0.14	**1.09 ± 0.29**	
	Lactic	3.45	0.31 ± 0.07	**0.95 ± 0.14**	-
	Benzoic	3.55	0.27 ± 0.13	0.44 ± 0.33	
	Acetic	4.35	0.26 ± 0.12	0.21 ± 0.22	
4.0 ± 0.1	Citric	3.65	0.28 ± 0.06	**2.78 ± 0.37**	0.15 ± 0.04
	Malic	4.55	0.24 ± 0.18	**2.08 ± 0.32**	0.10 ± 0.03
	Lactic	8.00	0.27 ± 0.18	**0.94 ± 0.28**	0.15 ± 0.04
	Benzoic	10.00	0.57 ± 0.58	**1.11 ± 0.27**	0.02 ± 0.03
	Acetic	20.00	0.22 ± 0.08	0.42 ± 0.29	0.11 ± 0.10

For each specific pH, different concentrations of EDTA, citric, malic, lactic, benzoic, and acetic acid (given in mM) are used in combination with 2 μM of ABgp46 to reduce E. coli O157:H7 cells in presence or absence of 5 mM of $MgCl_2$. Averages ± standard deviations for all experiments are given for n = 4 repeats.Marked in bold are ≥1 log reduction units observed.

and acetic acid), reaching maximum activity at pH of 4.0. It is known that some weak organic acids (e.g., citric, malic, benzoic, and lactic acid) have, to a lesser extent, chelating properties, but the additional acidity can also contribute to OM disruption (Alakomi et al., 2000; Theron and Lues, 2011). LPS disintegration is accomplished by the ability of undissociated acid groups to interact/pass through the negatively charged LPS (whereas negatively charged acid forms are repulsed) and migrate inside the cells to cause sub-lethal injuries. Intracellularly, the organic acid meets a higher internal pH (pH 7.2) and dissociates to produce protons (that can exit through specific proton channels) and anions (Breidt et al., 2004). Because the following bactericidal decreasing effect was observed: citric acid (pKa 3.13) > malic acid (pKa 3.4) > lactic acid (pKa 3.86) ≈ benzoic acid (pKa 4.19) > acetic acid (pKa 4.76) when combined with ABgp46, we speculate that this it is a reflection of acid dissociation constant (pKa) increase. This is somehow unexpected, since increasing the pKa favors undissociated groups that could penetrate into the cytoplasmic membrane potentiating cell damage. In contrast, acids with low pKa values would produce more hydrogen ions (that are not able to internalize) when exposed to an aqueous environment. Nevertheless, a similar trend was observed when organic acids (with lower pKa values) were incubated with medium-chain fatty acids (Kim and Rhee, 2013). These authors hypothesized that the medium-chain fatty acid action interact with the bacterial cell membranes, causing sub-lethal injuries. This would lead hydrogen ions to pass into the cell and result in a marked bactericidal effect. Perhaps a similar mechanism explains why ABgp46/citric acid and ABgp46/malic acid are more powerful combinations compared to other OMPs. The fact that no bacterial effect is observed in presence of 5 mM of $MgCl_2$, also suggests that free magnesium ions strengthen the electrostatic interactions between neighboring LPS components, avoiding acid entry and sequential sublethal damage (Vaara, 1999).

TABLE 3 | Combinatorial antibacterial activity of the best ABgp46/outer membrane permeabilizers (EDTA, citric, and malic acid) formula against broad range of planktonic Gram-negative pathogens.

Bacterial species	ABgp46/EDTA	ABgp46/citric	ABgp46/malic
E coli O157:H7	0.32 ± 0.15	**2.78 ± 0.37***	**2.08 ± 0.32***
A. baumannii #2	**>5.00†**	**>5.00†**	**>5.00†**
P. aeruginosa PAOI	**4.35 ± 0.20**	**4.25 ± 0.31**	**4.51 ± 0.22**
S. typhimurium LT2	0.57 ± 0.20	**4.24 ± 0.39***	**4.07 ± 0.37***
C. sakazakii CECT 858	**1.21 ± 0.38**	**2.05 ± 0.27**	**0.94 ± 0.23**
K. oxytoca A1CC 13182	0.26 ± 0.08	**0.96 ± 0.35***	0.81 ± 0.12*

Averages ± standard deviations for all experiments are given for n = 4 repeats. Marked in bold are ≥1 log reduction units observed. Statistically significantly (Student's t-test and P < 0.05) conditions between ABgp46/EDTA and ABgp46/organic acids are indicated with asteristic. †The detection level of 10 CFU/mL has been reached.

From preliminary testing, citric acid and malic acid were chosen for further testing against a larger group of G^- cells and compared with EDTA. ABgp46/EDTA revealed to be the only combination efficient against *Pseudomonas* and *Acinetobacter* cells, the same pattern was previously observed with Lys68 (Oliveira et al., 2014). This can be explained by the high phosphate content and consequently a higher concentration of stabilizing divalent cations present in the OM of these bacteria, compared with *Enterobacteriaceae* (such as *E. coli* and *S. typhimurium*; Nikaido, 2003), that are therefore, more prone to a chelation effect. The antibacterial effect of ABgp46 is more broaden in the presence of organic acids that are predicted

to destabilize the bacterial OM by an acidic effect, probably by lowering the bacterial internal pH and the accumulation of toxic substances (sub-lethal injury; Alakomi et al., 2000; Theron and Lues, 2011).

In summary, the structural and enzymatic characterization of ABgp46 provides novel insights into G^--like endolysins, and indicated that ABgp46 is an effective endolysin against several multidrug resistant pathogens. This technology can be appealing as a therapeutic/disinfectant agent for a range of applications. For instance, the use of topical solutions (e.g., cream or lotions) containing the endolysin/OMP formula could be useful for treating skin and soft tissue infections, associated with acne or chronic wounds.

AUTHOR CONTRIBUTIONS

LK, FS, and JA conceived the study. LK, RL, SS, and JA analyzed data. HO, DV, and SM performed experiments. HO and SM wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00208

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Enhanced Antibacterial Activity of *Acinetobacter baumannii* Bacteriophage ØABP-01 Endolysin (LysABP-01) in Combination with Colistin

*Rapee Thummeepak[1], Thawatchai Kitti[2], Duangkamol Kunthalert[1,3] and Sutthirat Sitthisak[1,3]**

[1] Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand, [2] Faculty of Oriental Medicine, Chiang Rai College, Chiang Rai, Thailand, [3] Centre of Excellence in Medical Biotechnology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand

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***Correspondence:**
Sutthirat Sitthisak
sutthirats@nu.ac.th

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Endolysins are lytic enzymes produced by bacteriophages with their ability to degrade the cell wall of bacterial hosts. Endolysin (LysABP-01) from *Acinetobacter baumannii* bacteriophage ØABP-01 was cloned, overexpressed and characterized. Endolysin LysABP-01 has a globular structure consisting of lysozyme-like (*N*-acetyl-β-D-muramidase) catalytic domain. It contains 185 amino acids which correspond to a 21.1 kDa protein. The lytic activity of the recombinant endolysin protein was determined by a plate lysis assay for its ability to lyse the autoclaved cell (crude cell wall) of the different bacterial species. LysABP-01 can degrade the crude cell wall of *A. baumannii* strains, *Escherichia coli* and *Pseudomonas aeruginosa* but not of *Staphylococcus aureus*. The antibacterial activity of LysABP-01 and its synergism with various antibiotics were tested. The results exhibited elevated antibacterial activity in a combination of the sub-MIC LysABP-01 and colistin. The checkerboard assay for measuring antibiotic synergy of LysABP-01 and colistin was performed. This combination was synergistic against various drug-resistant strains of *A. baumannii* (FIC index < 0.5). In summary, our study highlights the ability of LysABP-01 endolysin to hydrolyze the *A. baumannii* cell wall and its synergistic interaction with colistin.

Keywords: bacteriophage, *Acinetobacter baumannii*, endolysin, colistin

INTRODUCTION

Acinetobacter baumannii has emerged as a clinically significant pathogen that resistance to most available antibiotics. The increasing prevalence of multidrug-resistant *A. baumannii* (MDRAB) and extensively drug-resistant *A. baumannii* (XDRAB) infections has been reported worldwide (Perez et al., 2007). To date, colistin and tigecycline remain the most active antibiotics against drug-resistant *A. baumannii* (Henwood et al., 2002; Falagas and Kasiakou, 2005). However, the utilization of these drugs is limited due to high rates of toxicity and development of resistance (Falagas and Kasiakou, 2005; Navon-Venezia et al., 2007; Cai et al., 2012). Therefore, alternative antibacterial agents for treatments of MDRAB and XDRAB are urgently required.

Endolysins are lytic enzymes produced by bacteriophages during the last step of their replicative cycle. The enzymes degrade the cell wall of bacterial hosts and lead to cell lysis and phage progeny release (Young, 1992). Endolysins are classified as a new class of antimicrobials for the treatment of drug-resistant bacterial infection because of their rapid action, low evidence of resistance development and low cytotoxicity against mammalian cells (Schmelcher et al., 2012). A number of studies have demonstrated the efficiency of recombinant phage endolysins against gram-positive pathogens *in vitro* and in animal models. In contrast, applications of endolysins specific to gram-negative bacteria are limited because the outer cell membrane (OM) prevents exogenously applied endolysins from attracting the peptidoglycan layer (Fischetti, 2010; Schmelcher et al., 2012). Thus, many studies have focused on the enhancement of OM permeability using chelators, weak organic acids or high hydrostatic pressure (Briers et al., 2008, 2011; Oliveira et al., 2014, 2016). The chelator EDTA is very useful as an OM permeabilizer because of its ability to destabilize the lipopolysaccharide structure (Briers et al., 2011). However, the potential for future applications of an endolysin-EDTA combination is limited only for the topical treatment of localized bacterial infections, such as burn wound, eye and ear infections. The use of this combination is not suitable for the treatment of systemic infections as EDTA inhibits the blood clotting at the low concentration (Triantaphpyllopoulos et al., 1955). Combinations of the lytic enzyme with other antibacterials may produce synergistic effects or reduce the dose of a single agent (Djurkovic et al., 2005).

We previously characterized ØABP-01, a lytic phage which infects MDRAB strains (Kitti et al., 2014). We found that the ØABP-01 genome contains the endolysin encoding gene. In this study, we examined the antibacterial activity of LysABP-01 alone and in combination with conventional antibiotics against both MDRAB and XDRAB strains.

MATERIALS AND METHODS

Bacteriophage, Bacterial Strains, Plasmids and Growth Conditions

Bacteriophage, bacterial strains and plasmids used in this study are listed in **Table 1**. *A. baumannii* strain AB 1589 was used as the host for the ØABP-01 phage propagation. Four additional isolates were collected from difference hospitals in Thailand (**Table 2**). All isolates were identified on the species level by using biochemical tests and the PCR detection of the bla_{OXA-51} gene (Brown et al., 2005). All bacterial strains were grown in Luria-Bertani (LB) broth or agar (Hardy Diagnostics, Santa Maria, CA, USA) at 37°C.

Cloning of *lysABP-01*

Bacteriophage ØABP-01 genomic DNA was prepared according to the method reported in our previous study (Kitti et al., 2014). The endolysin gene *lysABP-01* (accession no. KF548002) was PCR amplified by using the phage DNA as a template with primers EndolysinABP-F and EndolysinABP-R (**Table 1**) which generated a 558 bp PCR product flanked by the restriction sites *Bam*HI and *Eco*RI. The resulting PCR product was cut with *Bam*HI/*Eco*RI restriction enzymes and cloned into the plasmid pBluescript II (pBluescript-*lysABP-01*). The *lysABP-01* in the pBluescript II was then subcloned into pRSETa expression vector (pRSET-*lysABP-01*). All initial DNA cloning procedures were carried out in *Escherichia coli* DH5α and then transformed into *E. coli* BL21 (DE3) pLysS. Restriction digestions and sequencing were used to verify the integrity of the cloned fragment.

Overexpression and Purification of LysABP-01

Log phase culture of *E. coli* BL21 (DE3) pLysS containing pRSET-*lysABP-01* ($A_{600} \sim 0.5$) was induced by the addition of IPTG to the final concentration of 1 mM. After incubation for 4 h at 37°C, cells were pelleted, washed and frozen at −80°C. The expressed protein was found in the insoluble cellular fraction, and was thus purified from the inclusion bodies. Thawed cells were resuspended in lysis buffer (145 mM NaCl, 20 mM Tris-Cl, pH 7.4) and disrupted by sonication with a ultrasonic cell disrupter (Sonic & Material Inc, Newtown, CT, USA). The soluble fraction was removed by centrifugation (5,000 × *g*). The pellets containing the inclusion bodies were solubilized in binding buffer containing urea (6 M urea, 500 mM NaCl, 20 mM Tris-Cl, 5 mM imidazole, pH 7.9). The solubilized recombinant protein was purified on the affinity chromatography column (His·Bind Kits, Novagen, Germany) under denaturing condition according to the manufacturer's instructions. The purified protein fractions were pooled and then diluted with four times volume of renaturation buffer (PBS containing 0.5 mM PMSF, 0.3 M arginine, 1% glycerol). The diluted protein was dialyzed against dialysis buffer (PBS containing 1 M urea, 1% glycerol) for overnight and then dialyzed against storage buffer (PBS containing 1% glycerol). The protein obtained after dialysis was concentrated using the vivaspin500 concentrator (GE Healthcare, UK). The purity of protein was checked by 12% SDS-PAGE, followed by staining of the SDS-PAGE gels with coomassie brilliant blue G-250 and the Pierce 6xHis Protein Tag Stain Reagent Set (Thermo sciencetific, USA). Protein concentrations were determined by the Bio-Rad protein assay (BioRad, Hercules, CA, USA).

Antibiotic Susceptibility Testing

The disk diffusion method was performed for all *A. baumannii* isolates to determine the susceptibility of amikacin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), tetracycline (30 μg), cefoperazone/sulbactam (105 μg), piperacillin/tazobactam (100/10 μg), colistin (10 μg) and tigecycline (15 μg) (Oxoid discs, UK). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute [CLSI], 2014). The antibiotic resistance profile was classified as multidrug-resistant *A. baumannii* (MDRAB) or extensively drug-resistant *A. baumannii* (XDRAB) based on a previously published description (Magiorakos et al., 2012).

TABLE 1 | Bacteriophage, bacterial strains, plasmids and primers used in this study.

Strains, phage, plasmids, or primers	Relevant characteristic(s), function, or sequence	Source or reference
Bacteriophage		
ØABP-01	*A. baumannii* phage from the waste water treatment plant	Kitti et al., 2014
Bacterial strains		
ATCC 19606	*A. baumannii* reference strain	ATCC
AB 1589	MDRAB strain, host for ØABP-01	Kitti et al., 2015
DH5∝	*E. coli* strain used for cloning	Novagen, Germany
BL21 (DE3) pLysS	*E. coli* strain used for protein overexpression, Cam^r	Novagen, Germany
ATCC 25922	*E. coli* reference strain	ATCC
ATCC 27853	*Ps. aeruginosa* reference strain	ATCC
ATCC 6538	*S. aureus* reference strain	ATCC
Plasmids		
pBluescript II	DNA cloning and sequencing vector, Amp^r	Fermentas, USA
pRSETa	Overexpression vector, Amp^r	Invitrogen, USA
pBluescript-*lysABP-01*	pBluescript II containing the *lysABP-01*	This study
pRSET-*lysABP-01*	pRSETa containing the *lysABP-01*	This study
Primers		
EndolysinABP-F	5′-GC*GGATCC*ATGATTCTGACTAAAGACGGATTTAGTATT-3′	This study
EndolysinABP-R	5′-GC*GAATTC*CTATAAGCTCCGTAGAGCACGTTC-3′	
bla_{OXA-51}-F	5′-TAATGCTTTGATCGGCCTTG-3′	Brown et al., 2005
bla_{OXA-51}-R	5′-TGGATTGCACTTCATCTTGG-3′	

TABLE 2 | Characteristics of clinical *A. baumannii* strains and their susceptibility to the LysABP-01 alone and in combination with colistin.

Strains ID	Year of isolation/Hospital	Drug resistance type	$MIC_{colistin}$ (μg/ml)		$MIC_{LysABP-01}$ (μM)		FICI
			Alone	Combined	Alone	Combined	
AB 1589	2007/H1	MDRAB	1	0.125	20	1.250	0.188
AB 0022	2013/H2	XDRAB	2	0.250	20	1.250	0.188
AB 0140	2014/H3	XDRAB	1	0.125	10	0.3125	0.156
AB 0269	2014/H4	XDRAB	1	0.125	10	0.625	0.188
AB 0405	2015/H5	XDRAB	0.5	0.0625	10	0.3125	0.156

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations was determined by the CLSI recommended broth microdilution techniques in Mueller-Hinton broth (HiMedia) (Clinical and Laboratory Standards Institute [CLSI], 2014). The antibiotics; ciprofloxacin, imipenem, colistin, chloramphenicol, gentamycin, erythromycin and tetracycline obtained from Sigma–Aldrich (St. Louis, MO, USA) were used. The MIC was defined as the lowest concentration of antibiotic that inhibit the bacterial growth. All MIC experiments were performed in duplicate and used *E. coli* ATCC 25922 as a quality control strain. The MIC value was used to calculate sub-MIC values (0.25 × the MIC) for the next experiment.

Plate Lysis Assay

Plate lysis assay was performed as described previously (Donovan et al., 2006) with some modifications. Bacterial cells of *A. baumannii* ATCC 19606, *A. baumannii* AB 1589, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 or *Staphylococcus aureus* ATCC 6538 in mid log phase were collected, washed once and suspended in PBS. The suspension was then autoclaved and centrifuged. The resulting pellet was resuspended in PBS (2% [vol/vol] of initial culture volume) and used as the substrate. Ten microliters of purified LysABP-01 (10 μM) was spotted onto the agar plate (1.5%) containing the substrate (5%). An equal volume of 10 μM egg white lysozyme was used as a positive control whereas PBS and storage buffer were used as negative controls. The spotted plates were incubated at room temperature.

Antibacterial Activity

Bacterial growth inhibition assay was performed as described previously (Knezevic and Petrovic, 2008) with little modifications. Bacterial colonies were transferred to Mueller Hilton broth (HiMedia) and cultured at 37°C for 4 h to reach mid-log phase. From these cultures, the turbidity of cell suspensions were adjusted to an equivalent 0.5 McFarland standard as measured by absorbance (0.08–0.1 at 625 nm), corresponding to approximately 10^8 CFU/ml. The adjusted cell suspensions were diluted 1:100 in double strengthen Mueller Hilton broth, and 50 μl was inoculated into each well (a final cell density $\sim 5 \times 10^5$ CFU/ml) in a microplate. The inoculum density was confirmed by plate count. Appropriate dilutions

of the LysABP-01 in PBS (25 μl) were added into wells, and the same volume of PBS or antibiotic (0.25 × the MIC) was added. Sample-inoculated microplates were incubated at 37°C overnight. After incubation, 25 μl of 0.1% sterilized TTC (Sigma Chemical Co, Saint Louis, MO, USA) was added into each well and incubated for additional 3 h. The absorbance was read at 540 nm using a Synergy 2 multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA). Each test was performed in triplicate.

Checkerboard Synergy Testing

Interactions between LysABP-01 and the selected antibiotic were assessed by the checkerboard broth microdilution method as previously described (Pillai et al., 2005). Testing was performed in Mueller-Hinton broth, with a final inoculum of 5×10^4 CFU per well in microplates. LysABP-01 and the selected antibiotic were diluted twofold horizontally and vertically, respectively. The concentrations of the selected antibiotic and LysABP-01 ranged from 0.0625 – 2 × MIC and 0.0156 – 2 × MIC, respectively. Microplates were incubated overnight at 37°C. After incubation, 25 μl of 0.1% sterilized TTC (Sigma Chemical Co, Saint Louis, MO, USA) was added into each well and incubated for 3 h. Test results were used to calculate the fractional inhibitory concentrations (FICs) and FIC index (FICI). FICs of LysABP-01 and the selected antibiotic were plotted on the x/y plot to generate an isobologram. A FICI was interpreted as follows: ≤0.5, synergy; >0.5 – ≤1.0, additive; >1.0 – ≤ 2.0, indifference; and >2.0, antagonism (Pillai et al., 2005).

FIGURE 1 | Amino acid sequence alignment of LysABP-01. The sequences used for alignment analysis were LysAB2 (from phage ϕAB2, accession no. ADX62345), PlyAB1 (from phage Abp1, accession no.YP_008058242) and LysABP-01 (from phage ØABP-01, accession no. AHG30899). Eleven mutation residues are indicated by asterisks (*), five of which are found in conserved domain at positions 96, 100, 103, 108, and 111.

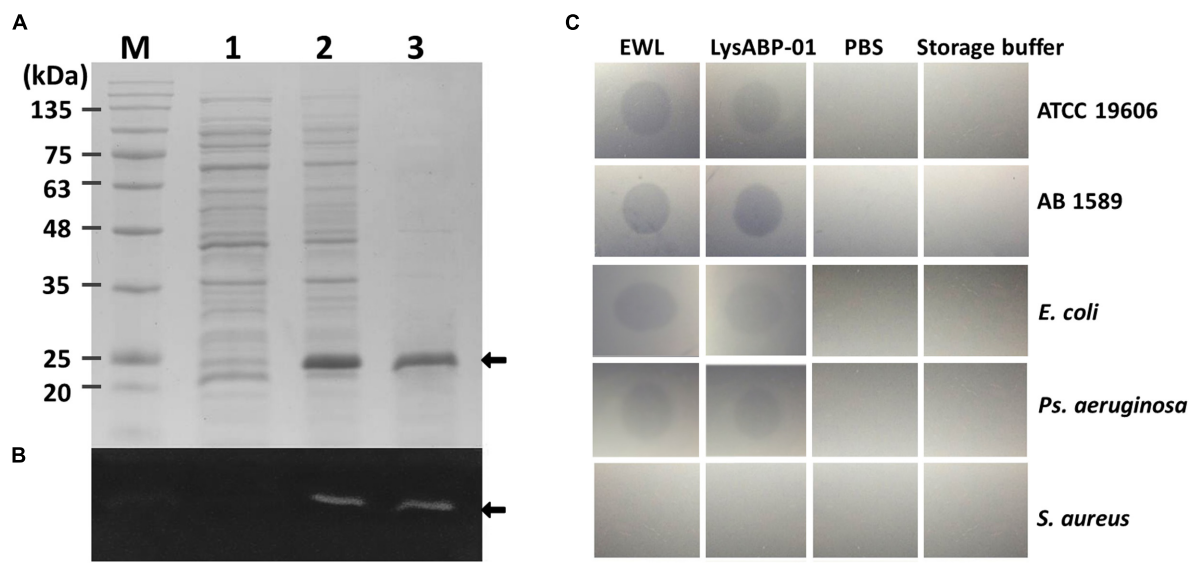

FIGURE 2 | Analysis of histidine-tagged LysABP-01. (A) SDS-PAGE analysis for expression and purification of LysABP-01. **(B)** Fluorescent stain for detection of His_6-tagged LysABP-01 on a SDS-PAGE gel. Lane M, BLUeye prestained protein ladder; Lanes 1, un-induced bacterial lysate; Lane 2, IPTG-induced bacterial lysate; Lane 3, the purified Lys-ABP-01 after dialysis. **(C)** Plate lysis assay of the purified LysABP-01. Egg white lysozyme (EWL), *A. baumannii* ATCC19606 (ATCC 19606), *A. baumannii* AB 1589 (AB 1589), *E. coli* (ATCC 25922), *Ps. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 6538).

RESULTS

Characterization of the LysABP-01

The endolysin gene of the phage ØABP-01, named *lysABP-01*, has been previously detected and sequenced (accession no. KF548002). The LysABP-01 contains 185 amino acid residues, which corresponds to a 21.1 kDa protein. Conserved domain analysis using the Pfam database has revealed the presence of a lysozyme-like (*N*-acetyl-β-D-muramidase) catalytic domain between residues 75 and 128 of LysABP-01. A ClustalW2 alignment of LysABP-01 with two phage endolysins showed high similarity in the conserved domain region (**Figure 1**). Although these three endolysins share high sequence similarity, 5 out of 11 amino acid mutations were found in the conserved region among them (**Figure 1**).

Over-Expression, Purification, and Lytic Activity of LysABP-01

After induction with IPTG, the expressed LysABP-01 was detected by SDS-PAGE analysis (**Figure 2A**). The presence of a histidine-tag in the expressed recombinant proteins was confirmed by staining with the Pierce 6xHis Protein Tag Stain Reagent Set (**Figure 2B**). We found that most of the expressed LysABP-01 was insoluble protein. The insoluble protein fraction was solubilized and purified using affinity chromatography. The purified LysABP-01 was refolded and concentrated. The lytic activity and spectrum of LysABP-01 were tested using the plate lysis assay and autoclaved cells (crude cell wall) from the log phase cells of gram-positive and gram-negative bacteria, which were used as substrates. The results showed that LysABP-01 had strong lytic activity toward two *A. baumannii* strains (ATCC 19606 and AB 1589) and weaker activity against reference strains of *E. coli* and *Ps. aeruginosa* (**Figure 2C**). In addition, no lysis was detected in a gram-positive strain, *S. aureus* (**Figure 2C**).

Antibacterial Activity of LysABP-01 and Its Synergism with Antibiotics

The MIC of purified LysABP-01 was tested using bacterial growth inhibition assays for its ability to inhibit the growth of viable cells of the MDRAB strain. The results indicated that the LysABP-01 can prevent the growth of AB1589 with a MIC of 20 μM (**Figure 3**; **Table 2**). The interactions between LysABP-01 and seven antibiotics were screened by growth inhibition assay at 0.25 × the MIC of two agents (**Supplementary Table S1**). As shown in **Figure 4A**, only the combination of LysABP-01 plus colistin exhibited elevated antibacterial activity (nearly 100% of growth inhibition rate). In order to verify these results, we performed a checkerboard assay for measuring antibiotic synergy. The FICI value for the combination of LysABP-01 and colistin (0.0625/0.125 × the MIC) was determined as 0.188, which indicates synergism (**Table 2**). Test results were also represented by the isobologram generated by plotting the FICs of LysABP-01 and colistin. The isobologram is presented in **Figure 4B** and it shape being concave, suggests that the two antimicrobials had a synergistic effect against the representative MDRAB strain.

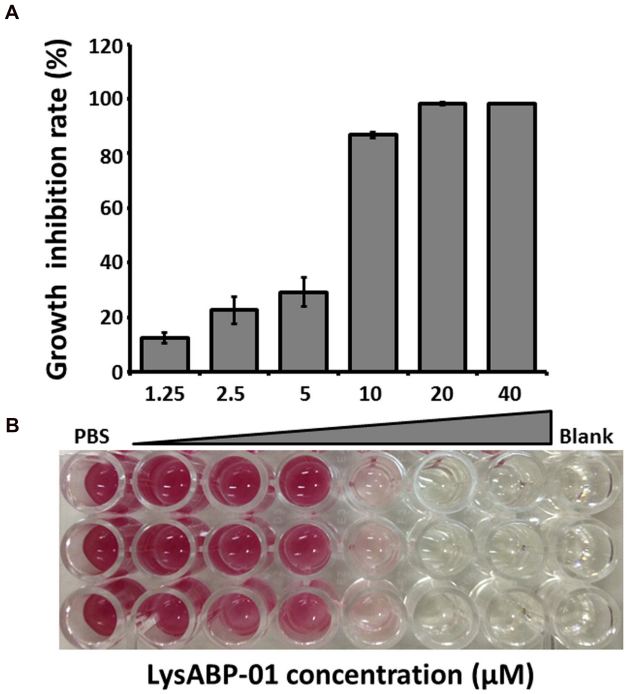

FIGURE 3 | Antibacterial activity of LysABP-01. (A) Bacterial growth inhibition activity of varying concentrations of LysABP-01. The bar graph depicts the percent growth inhibition and represents the mean percentage ± SD of triplicate experiments. **(B)** The representative microplate image of TTC staining.

The Susceptibility of XDRAB Strains to LysABP-01 Combined with Colistin

The selected strains of *A. baumannii* were obtained from various hospitals located in different areas of Thailand. All four isolates were positive for the species-specific bla_{OXA-51} gene (data not shown). The results from the disk diffusion method revealed that four *A. baumannii* isolates were resistant to all tested antibiotics except colistin and tigecycline. Thus, these strains were classified as XDRAB (**Table 2**). The *in vitro* activity of LysABP-01 against a broad range of *A. baumannii* strains was studied. The results of the antimicrobial susceptibility testing of different *A. baumannii* strains to LysABP-01 alone and in combination with colistin are shown in **Table 2** and **Supplementary Figure S1**. By a checkerboard test, the FICI and isobologram of the colistin-LysABP-01 combination showed synergistic against all tested XDRAB strains (**Table 2**; **Supplementary Figure S1**).

DISCUSSION

The emergence of drug-resistant bacteria is a therapeutic problem. Bacteriophages and their endolysins have been

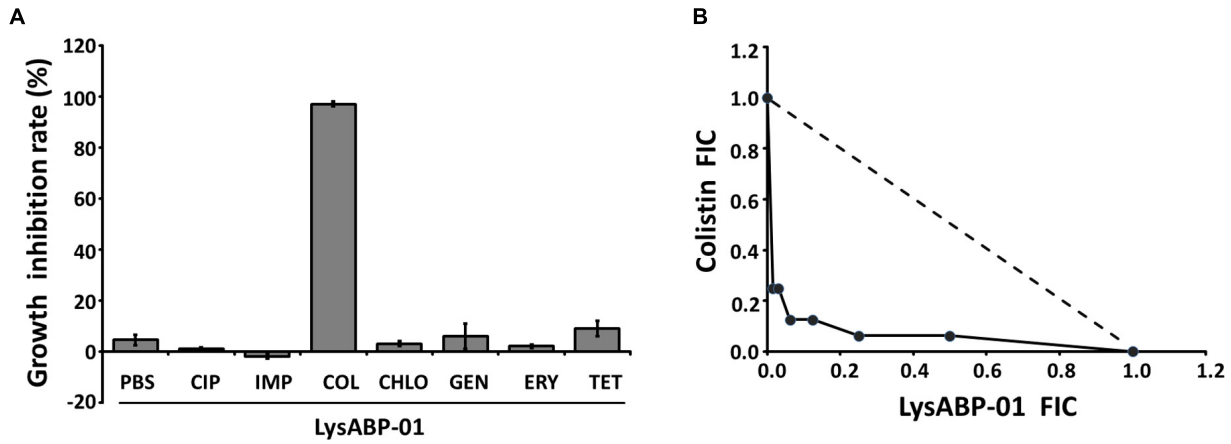

FIGURE 4 | The results of screening and confirmatory testing for synergistic interaction. (A) The growth inhibition rate (the bar graph) of LysABP-01 combined with seven antibiotics. Data are expressed as the mean percentage ± SD of triplicate experiments. Phosphate buffer saline (PBS), ciprofloxacin (CIP), imipenem (IMP), colistin (COL), chloramphenicol (CHLO), gentamycin (GEN), erythromycin (ERY) and tetracycline (TET). **(B)** The isobologram of the checkerboard assay. FIC values derived from combinations of LysABP-01 and colistin were used to plot the isobologram. The dashed line illustrates the theoretical additive interaction between two agents.

recognized as alternative therapeutic compounds for combating drug-resistant bacterial infections. In our previous work, three phages infecting MDRAB were isolated and characterized (Kitti et al., 2014). We found the ØABP-01 phage belonged to *Podoviridae* family. This phage had shown a good lytic activity on MDRAB and its genome contains the endolysin gene, named *lysABP-01*, which was further studied. *In silico* sequence analysis revealed that the LysABP-01 was conserved in other endolysins from Acinetobacter phage such as LysAB2, PlyAB1 and ABgp46 (Lai et al., 2011; Huang et al., 2014; Oliveira et al., 2016). The lysozyme-like catalytic domain of LysABP-01 is a *N*-acetyl-β-D-muramidase that cleaves β-1, 4-glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan (Schmelcher et al., 2012). As illustrated in **Figure 1**, 11 mutation residues among three endolysins, especially five points in the conserved domain, may contribute to the enzyme's catalytic function.

The recombinant LysABP-01 was located in the insoluble fraction as inclusion bodies due to its toxicity to host cells (García et al., 2010). We have successfully purified and refolded LysABP-01 from inclusion bodies. The result from plate lysis assay revealed that the recombinant LysABP-01 was able to digest crude cell wall from *A. baumannii* reference and MDRAB strains. This result was consistent with the findings from conserved domain analysis. However, LysABP-01 was active against other gram-negative bacteria but not able to hydrolyze crude cell walls of gram-positive bacteria, which corresponded with previous reports (Lim et al., 2014; Oliveira et al., 2014).

Enzyme activity of LysABP-01 was not only active against crude cell wall but also able to inhibit the growth of viable cells. The antibacterial activity of LysABP-01 was in agreement with the report of Lai and co-workers (Lai et al., 2011). In their study, they found that the presence of 500 μg/ml of the LysAB2 could reduce the cell viability of *A. baumannii* to less than 1% (Lai et al., 2011), while in our study we found that the presence of 20 μM (∼500 μg/ml) of LysABP-01 inhibited the growth of *A. baumannii*. Gram negative bacteria have an OM that can protect peptidoglycan from the direct contact of lytic enzymes. Thus, the combination approach can be used to increase the efficacy of lytic enzymes. Previous studies have reported that the combination of colistin with other antimicrobials, such as vancomycin (Gordon et al., 2010), teicoplanin (Wareham et al., 2011), and daptomycin (Galani et al., 2014) produced synergistic effects against MDRAB strains. Because only the combination of LysABP-01 and colistin was found to increase antibacterial activity (**Figure 4B**), the checkerboard assay was not carried out in all combinations. The *in vitro* combination of colistin with LysABP-01 in this study revealed a synergistic interaction against MDRAB, AB 1589. We also found that this protein is effective against a broad range of XDRAB for both exclusive and in combination treatments. Moreover, the MICs of LysABP-01 were reduced up to 32-fold, while the MICs of colistin were reduced up to eightfold in the combination (**Table 2**). Colistin, also called polymyxin E, is a cyclic lipopeptide antibiotic consisting of a cationic peptide ring and a lipophilic fatty acid tail. The positively charged molecules of this drug interact with the negatively charged lipid A phosphates and displace the divalent cations (Mg^{2+} and Ca^{2+}) in the lipopolysaccharide, which is essential for the OM stability. This interaction causes OM damage and leakage of cellular components leading to cell death (Falagas and Kasiakou, 2005). The cell membrane destabilizing activity of colistin could be exploited to promote the penetration of the endolysin through the OM toward its target in the cell wall. Although polymyxins are often the most effective drugs for MDRAB and XDRAB treatment, the use of these drugs in clinical practice is very restricted because of their effects

on resistance development and nephrotoxicity or neurotoxicity (Falagas and Kasiakou, 2005; Cai et al., 2012). In this work, we have demonstrated that the colistin-LysABP-01 combination can produce synergy with a wide range of strains, reduce the dose of two agents, and may decrease the toxicity of colistin. Thus, the synergistic results illustrate the potential for future therapeutic use of this combination.

CONCLUSION

This study shows that LysABP-01 contains antibacterial activity against *A. baumannii*. This enzyme has a synergistic interaction with colistin, an antibiotic with a directed action against the bacterial cell membrane. The implication of this study is that LysABP-01 will work as an alternative agent in combination with colistin against *A. baumannii*.

AUTHOR CONTRIBUTIONS

Study conception and design: DK, and SS. Acquisition of data: RT. Analysis and interpretation of data: RT, and TK. Drafting of manuscript: RT, TK, and SS. Critical revision: RT, TK, and SS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01402

FIGURE S1 | *In vitro* synergistic activity of LysABP-01 combined with colistin against XDRAB strains. (A) The representative checkerboard plates. **(B)** The isobolograms representing the drug interaction of LysABP-01 plus colistin. FIC values derived from combinations of LysABP-01 and colistin were used to plot the isobologram. The dashed line illustrates the theoretical additive interaction between two agents.

TABLE S1 | MICs of conventional antibiotics against *A. baumannii* AB 1589.

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frontiers in Microbiology

REVIEW
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Modular Approach to Select Bacteriophages Targeting *Pseudomonas aeruginosa* for Their Application to Children Suffering With Cystic Fibrosis

Victor Krylov[1], Olga Shaburova[1], Elena Pleteneva[1], Maria Bourkaltseva[1], Sergey Krylov[1], Alla Kaplan[1], Elena Chesnokova[1], Leonid Kulakov[2], Damian Magill[2] and Olga Polygach[1]*

[1] Laboratory for Genetics of Bacteriophages, Department of Microbiology, I.I. Mechnikov Research Institute for Vaccines and Sera, Moscow, Russia, [2] Medical Biology Centre, School of Biological Sciences, Queen's University Belfast, Belfast, UK

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Laurent Debarbieux, Institut Pasteur (INSERM), France

***Correspondence:**
Victor Krylov
krylov.mech.inst@mail.ru

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This review discusses the potential application of bacterial viruses (phage therapy) toward the eradication of antibiotic resistant *Pseudomonas aeruginosa* in children with cystic fibrosis (CF). In this regard, several potential relationships between bacteria and their bacteriophages are considered. The most important aspect that must be addressed with respect to phage therapy of bacterial infections in the lungs of CF patients is in ensuring the continuity of treatment in light of the continual occurrence of resistant bacteria. This depends on the ability to rapidly select phages exhibiting an enhanced spectrum of lytic activity among several well-studied phage groups of proven safety. We propose a modular based approach, utilizing both mono-species and hetero-species phage mixtures. With an approach involving the visual recognition of characteristics exhibited by phages of well-studied phage groups on lawns of the standard *P. aeruginosa* PAO1 strain, the simple and rapid enhancement of the lytic spectrum of cocktails is permitted, allowing the development of tailored preparations for patients capable of circumventing problems associated with phage resistant bacterial mutants.

Keywords: choice of phage, Phage therapy organization, cystic fibrosis, phage–host interactions, *Pseudomonas aeruginosa*, modular phage compositions, personalized phage therapy

INTRODUCTION

The use of antibiotics in the treatment of bacterial infections increasingly encounters difficulties caused by the emergence and rapid spread of pathogenic bacteria exhibiting multidrug resistance. The discovery and study of new antibiotics, is an extremely expensive and arduous process, associated with many risks (Fowler et al., 2014). As a result there is renewed interest in phage therapy – the use of bacterial viruses (phages), in their role as "natural enemies" of the bacteria. The main objective of phage therapy is the elimination of pathogenic bacteria within the foci of infection. But following the emergence and multiplication of phage-resistant bacteria, the only way forward will be through the use of new phages capable of overcoming this resistance.

Phage therapy was proposed and implemented for treating bacterial infections by Felix D'Herelle, one of the pioneers of bacterial viruses about 100 years ago. Affirmation as to the potential medical application of bacteriophages has been demonstrated by their prolonged use in the treatment of certain bacterial infections in Russia, Georgia, and Poland. Indeed, there is particular promise with respect to both the efficacy and safety of phage therapy toward superficial infections of skin and mucus membranes (Abedon et al., 2011; Krylov, 2014). In these countries therapeutic mixtures active against a variety of bacterial pathogens are produced on an industrial scale, so a significant level of expertise and a large collection of bacteriophages have been accumulated over the years. However, widespread acceptance of phage therapy is far from being achieved. The use of phage therapy in Western countries mostly ceased following the introduction of antibiotics into medical practice but now, due to the emergence of pathogenic bacteria resistant to all available antibiotics the need for a revival seems almost inevitable.

Scientific professionals are well aware of the epidemic potential of rapidly spreading multidrug resistance. This understanding has led to a detailed discussion regarding the need to implement the use of phage therapy in the medical practices of different countries and in providing solutions to the outstanding problems associated with such practices (Alavidze et al., 2016). Among them are problems of a legislative nature, the need to prove the long term safety of phage therapy, promoting the recognition of phage therapy beyond a handful of Eastern European countries to an international level, issues with the production of phage cocktails on an industrial scale, the financing associated with this, and problems with patenting.

Whilst the need to address these problems is recognized, one should bear in mind that the future will bring with it cases in which phages may be the only route to antibacterial therapy. An example of such a situation could involve the appearance of plasmids conferring bacterial resistance to colistin in specialized centers for the treatment of patients with cystic fibrosis (CF), resulting in a significantly worsened prognosis. Therefore, the problems associated with the implementation of phage therapy for the control of multidrug resistant *Pseudomonas aeruginosa* requires an immediate solution. In cases where repeated infections are inevitable (e.g., in CF patients), and no appropriate antibiotics are available, phage therapy may prove to be the only option to prolong the life expectancy of patients.

In parallel to this work, we believe in making the study of phage therapy in children suffering from CF a priority area. Success in such cases will secure a place for phages in our medical arsenal by establishing a positive public perception for the treatment along with the experience that will be accumulated in the course of the studies.

Here various aspects required for the implementation of phage therapy for *P. aeruginosa* pulmonary infection in children suffering from CF are discussed in detail. In the age of growing antibiotic resistance, phage therapy may prove to be the only feasible route to antibacterial therapy.

FEATURES OF CYSTIC FIBROSIS DUE TO BACTERIAL INFECTION

Cystic fibrosis is a frequent hereditary disease of Caucasians. Various estimates have placed on average one out of every 2500 – 7000 newborns as being homozygous for the mutant allele CFTRΔF508. The pathogenesis of the disease is associated with impaired secretory function of the pulmonary epithelium, leading to the disruption of ion exchange and accumulation of mucus and fluid in the lungs. This creates favorable conditions for the growth of bacteria of various species. At the early stages, staphylococcal infection is the major culprit, followed by *P. aeruginosa* domination (Kosorok et al., 2001). The production of alginate by these bacteria, an extremely viscous polysaccharide, by these bacteria promotes the proliferation and survival of other hazardous species, such as *Burkholderia sp.,* worsening the overall prognosis (Henry et al., 1992; Lynch and Dennis, 2012). Symptoms of this disease may differ significantly amongst individuals but the major ones tend to be obvious from a young age.

During the course of infection a gradual change in the properties of the primary infecting strains of *P. aeruginosa* takes place, manifested by a decrease in their pathogenicity and virulence, as well as increased sensitivity to the lytic effect of bacteriophages (Friman et al., 2013; Cullen et al., 2015). Moreover these strains, being adapted to the conditions of the lungs, are influencing the expression of the pathogenic properties of other species in the concomitant microflora (such as *Burkholderia multivorans*, *Burkholderia cenocepacia, Pandoraea pulmonicola* and *Pandoraea apista*) significantly lowering them.

It has been proposed that such domination is accomplished through gene products involved in quorum sensing and pyoverdine biosynthesis (Costello et al., 2014). The gross activity of the set of interacting bacteria leads to the destruction of the lungs, generally seen as the major reason for the reduction in life expectancy of CF patients. The use of new antibiotics as well as inhalation protocols for their introduction into lungs has significantly increased patient life expectancy. However, the use of antibiotics, even in high doses does not always lead to the eradication of *P. aeruginosa* due to the persistence of some bacterial cells through their transformation into dormant cyst-like and non-culturable cells (Mulyukin et al., 2015).

An important factor in the evolution of *P. aeruginosa* in specialized CF treatment centers is a constant inclusion of new bacterial strains carrying different prophages in their genomes, including transposable phages through cross-infection of patients. It is believed that the activity of the transposable phages in pathogenic islands has led to the emergence of strains exhibiting enhanced virulence, pathogenicity and resistance to environmental factors and, as a consequence, the capability for epidemic spread (Winstanley et al., 2009). Such epidemic spread is also a risk factor outside the CF centers (Mohan et al., 2008).

The different approaches were proposed to extend the life of patients and to improve its quality. Among them is the surgical replacement of patient's lungs (Morrell and Pilewski, 2016). There was also hope to develop a drug for selective inhibition of alginate

synthesis in bacterial cells (Hershberger et al., 1995). Another proposition was to substitute the function of the mutant gene, by creating a viral integratable vector carrying the wild-type allele (Yan et al., 2015). There is also a suggestion that modification of the activity of toll-like receptors and other coreceptors with genetic engineering may lead to changes in critical components of CF immunobiology. It is not yet possible, but is expected that with improvements in bioengeneering, that the development of novel vectors and methods of delivery, biocompatibility, and safety, then therapeutic effectiveness will be successfully achieved. This however, could take many years (Atkinson, 2008). Thus, one must assume that at the present time, and in the immediate future, antibacterial therapy will be the major approach to treat CF patients. This currently takes the form of "aggressive antibiotic therapy," usually by alternating two antibiotics in order to reduce the probability of the occurrence and accumulation of multidrug-resistant mutants. In cases involving resistant strains arising, there is the option to utilize the inhalation of colistin as a last resort, a very toxic substance exhibiting a strong surface activity. Resistance to colistin due to mutations in the bacterial chromosome is a rare event because it requires mutations in two genes controlling the structure of the bacterial plasma membrane (Fernández et al., 2010; Lee et al., 2014), and there was a hope that the inhalation of colistin may be a reliable protective measure in adults (for children, in the light of its high toxicity, this is possible only after the child reaches 6 years age).

Recently, however, a new problem has arisen that may limit the use of colistin. A transmissible plasmid has been isolated encoding MCR-1, an enzyme that transforms the bacterial lipid A in the outer membrane to a colistin resistant state (Liu et al., 2015). This plasmid has been found in *Escherichia coli* strains, but given the relatively ease of interspecies migration for plasmids it is almost inevitable that a situation will arise when some strains of *P. aeruginosa* infecting patients with CF will acquire additional transmissible resistance to colistin. It is unclear yet corrected whether a strain with such a plasmid could be displaced by a more physiologically active but colistin-sensitive strain in the absence of the antibiotic selective pressure, as sometimes happens in the case of chromosomal resistance to colistin. Therefore, it is possible that the use of bacterial viruses – phage therapy – may not only be suitable, but also a valuable method of antibacterial therapy, especially in the treatment of children in specialized CF departments, under careful medical and microbiological control.

COMPARISON OF BACTERIOPHAGES AND ANTIBIOTICS AS ANTIMICROBIAL AGENTS: EVALUATION OF RESULTS IN THE APPLICATION OF PHAGE THERAPY

Bacteriophages acquire the properties of living systems in the process of infecting sensitive bacteria. There are different viewpoints about the relations between phages and their hosts. They are often considered enemies of bacteria, but one can also look at them from the perspective of being a potential source of additional genetic material designed to maintain the evolution of the bacterial domain and their adaptive potential. In either instance, their natural purpose is not for use in the treatment of human and animal diseases Thus, it is evident the desire do not use as therapeutics the phages with substantial potential to transduce bacterial genes. It is necessary to bear this in mind in the application of phage therapy and this is one reason why the outcome of such treatment can be of an unpredictable nature. Additionally, we cannot preclude the possibility of events such as recombination with related phages, the appearance of different phage resistant bacterial mutants, or the establishment of pseudolysogeny (Hobbs and Abedon, 2016) in sensitive bacteria, and interactions with different plasmids, temperate, and virulent phages of unrelated species. Knowledge of possible modifications in relations of virulent phages and bacterial hosts is very important for success of phage therapy. That requires prior conduction of *in vitro* comparative studies of the growth of different phages infecting different clinical isolates in microbiotas.

So how do we evaluate the effectiveness of phage therapy? When treating open infected wounds using bacteriophages, even a single use of an appropriately selected phage mixture is sometimes sufficient (Voroshiliova et al., 2013) for successful cleansing of the wound. Objective assessment of antibacterial phage therapy in the treatment of pulmonary infection can be made by comparing the changes in bacterial compositions in the sputum of patients before and after phage application (cited in Abedon, 2015), with evaluation of the resistance of surviving bacteria to the therapeutic phages utilized (see later). This is important, as the emergence of resistant variants provides a level of confirmation as to the success of the previous phage inhalation treatment, but on the other hand, demonstrates the need to continue treatment with the selection and introduction of new active mixtures of phages. Sometimes the effect of phage therapy can only be observed after several days to allow the accumulation of an active phage concentration sufficient to infect most pathogenic cells. In addition, evaluation of the effectiveness of phage therapy may be valid only in conditions preventing cross-infection of the patient with other strains that are currently circulating in the hospital.

PREREQUISITE FOR SAFE PHAGE THERAPY – REFUSING TO EXPAND THE LYTIC SPECTRUM OF PHAGE PREPARATIONS WITH RANDOM BACTERIOPHAGES

In Eastern European countries where phage therapy is officially recognized, the decision on its application is taken by medical practitioners. Factories located across Russia are producing phage preparations active against several regionally chosen species of pathogenic bacteria. With a certain periodicity, the phage preparations are going through a process of "adaptation" to novel circulating phage resistant strains derived from various specialized clinics and hospitals of the region by the introduction of new phages into them from external sources to overcome

this resistance. This method of enhancing the lytic spectrum of phage commercial preparations is simple, fast, inexpensive, and yet very effective (Voroshiliova et al., 2013). Even though the newly introduced phages have not been identified and their properties remain unexplored, these preparations show good results in the short-term treatment of various open infected wounds, intestinal, and urinary tract infections. The composition of the phage preparations having a similar designation, but produced by different manufacturers at different times, may differ in their activities. This non-identical nature of phage preparations of varying origin can allow for performance comparisons to be made and subsequently allow one to choose the most appropriate mixture. Generally speaking, however, doctors only consider the possibility of utilizing phage therapy after repeated failures with different antibiotics (and even then not always!). Nevertheless, the use of such preparations of random enrichment, containing uncharacterized phages, including temperate, transposable and filamentous ones potentially involved in promoting the evolution of bacterial pathogens in CF can lead to undesirable consequences (McCallin et al., 2013).

In the course of treating chronic infections, the introduction of new active phages should take place in real time, in order to prevent the occurrence and accumulation of new phage-resistant variants. It is advisable to consider the possibility of modifying the composition of a medicinal mixture, in which the probability of occurrence of such undesirable effects could be eliminated or substantially reduced. This would help transform phage therapy from an almost forgotten in official medicine procedure with limited use into an established method for antibacterial therapy (beyond the realms of CF). First of all, it is necessary to prohibit the expansion of the lytic spectrum when introducing a mixture of new phages with unexplored properties. Then, it would also be desirable that the procedure for adaptation of activity of the phage mixture be applied to the treatment of an individual patient and that this is carried out within a clinical setting. Such modification does not require the introduction of significant organizational changes, and this work could be accomplished by existing professional staff of the microbiology laboratory at the clinic that possess some practical experience working with bacteriophages. Their task will be to monitor whether phage-resistant strains arise in patients and to then select new phages from a phage bank to restore the therapeutic activity of the phage mixture.

Since in the absence of active antibiotics, phage therapy could become a permanent clinical procedure, it is important to keep in mind that some unpredictability can arise that could affect the outcome of each phage application. Bacteriophage multiplication in lungs may depend on many uncontrollable conditions – interactions of different species of bacteria, the nature of the infected surface, lung fluid composition and viscosity, amongst other variables.

A critical factor in order to support uninterrupted treatment time is the permanent expansion of the lytic spectrum of phage preparations. Therefore, it is of the utmost importance that rigorous monitoring procedures are established so as to hastily detect potential phage resistant mutants, as their rapid eradication may be concentration dependent. Up until now, the presently adopted procedure for expanding the range of lytic activity is ineffective, as adaptation demands too much time. Optimization of lytic spectra for phage therapy, however, should be personalized in real time. Naturally, it not only increases the demands on the reliability of the active phage preparation, but also transforms clinical phage therapy in the ongoing research. As an example - in medical preparations temperate phage and their lytic variants, even those possessing unique lytic spectra, should be absent. Indeed, potential transitions to the prophage state (as a result of recombination with phages already present in the lungs) can result in the imparting of properties such as heightened pathogenicity, increased stability in the environment, and capacity for epidemic spread.

We believe that an absolute prerequisite for the implementation of phage therapy worldwide as a method of antibacterial therapy in CF is the investigation of genetic and other properties of phages administered into the preparations and potential interactions associated with these. One can expect that such work in prospect will provide valuable new data required not only for the characterization of phages as antibacterials but also, for example, as the carriers of genetic material capable of compensating the CFTR gene mutation responsible for CF.

Numerous studies have been carried out with the end goal of achieving the resurrection of phage therapy within the Western world, including the treatment of CF associated infections. Various models of acute and chronic infection in mice, rats, wax moths, and cell culture provide an excellent framework for testing phages under a variety of conditions. For example, it has previously been demonstrated that the use of wax moths (*Galleria mellonella*) as a model allows one to carry out rapid comparisons of phage activity, useful for making swift evaluations of effectiveness with respect to particular phage preparations *in vivo* during the treatment process (Beeton et al., 2015; Olszak et al., 2015). Using a mouse model, it is also possible to estimate bactericidal activity by lytic phages in lung infection. One study has shown a good correlation of activity *in vitro* and *in vivo* for all virulent phages utilized, however, with the exception of two species, shown as being insufficiently active *in vivo* despite good *in vitro* activity (Henry et al., 2013). This is a significant observation as it highlighted the need to increase the lytic activity in phages of several species through the selection of specific mutants displaying enhanced virulence. This also demonstrates the need to develop optimal configurations for phage mixtures, so as to achieve the best treatment outcomes. Indeed, with respect to the utilization of multi phage preparations, the therapeutic activity is a product of the combined effect of all phages present. In fact, Alemayehu et al. (2012) previously demonstrated the ability to stop a primary infection of mice with a multidrug-resistant *P. aeruginosa* strain taken from a CF patient with lung infection with such phage preparations. The bactericidal effect of these phages has been confirmed with an *in vitro* infection of this strain on a CF biofilm bronchial epithelial (CFBE41o) cell line. In addition to all of this, application of two strain specific virulent phages in a murine model of acute pulmonary *P. aeruginosa* infection was accompanied not only by rapid elimination of the pathogen, but by a concomitant decrease in the level of

inflammation (Pabary et al., 2015). However, the requirement for continuous monitoring of lung composition in CF patients during the application of phage therapy is essential, as is the need to take additional measures in order to prevent the permanent adhesion of bacteria to the pulmonary epithelium. Friman et al. (2016) demonstrated that individual pre-adaptation of a phage for a different patient increases the efficiency of the phage killing effect. However, conducting this pre-adaptation process may take some time and delay the onset of the actual treatment, which is obviously not always possible. In light of all this research, it is clear that there is significant promise for the implementation of phage therapy in the treatment of CF associated infection and no doubt beyond this, however, rigorous protocols must be set in place if we are to ensure both the safety and maximum potential of treatment.

Significance of Temperate Bacteriophages in CF Clinical Manifestations

Studies using metagenomic analysis have shown how temperate bacteriophages of *P. aeruginosa* in clinical conditions following induction become involved in the horizontal transfer of DNA, and acquire selective preference for the development and accumulation in the specific conditions of the lower lung (Tariq et al., 2015). Their ability for transfer of additional genes increases with time during the deterioration of patient lung function and disease prognosis. This represents the first, direct clinical confirmation for the proposed evolution of phages *in vivo* at mucous lung surfaces. The active role of phage gene expression in the course of the disease and its prognosis has been confirmed in a study involving the Liverpool epidemic strain. Other research (Lemieux et al., 2016) has shown that groups of strains possessing mutations in prophage regions and pathogenic islands displayed reduced pathogenicity in a rat model of chronic lung infection and was associated with disturbances in phage transcription.

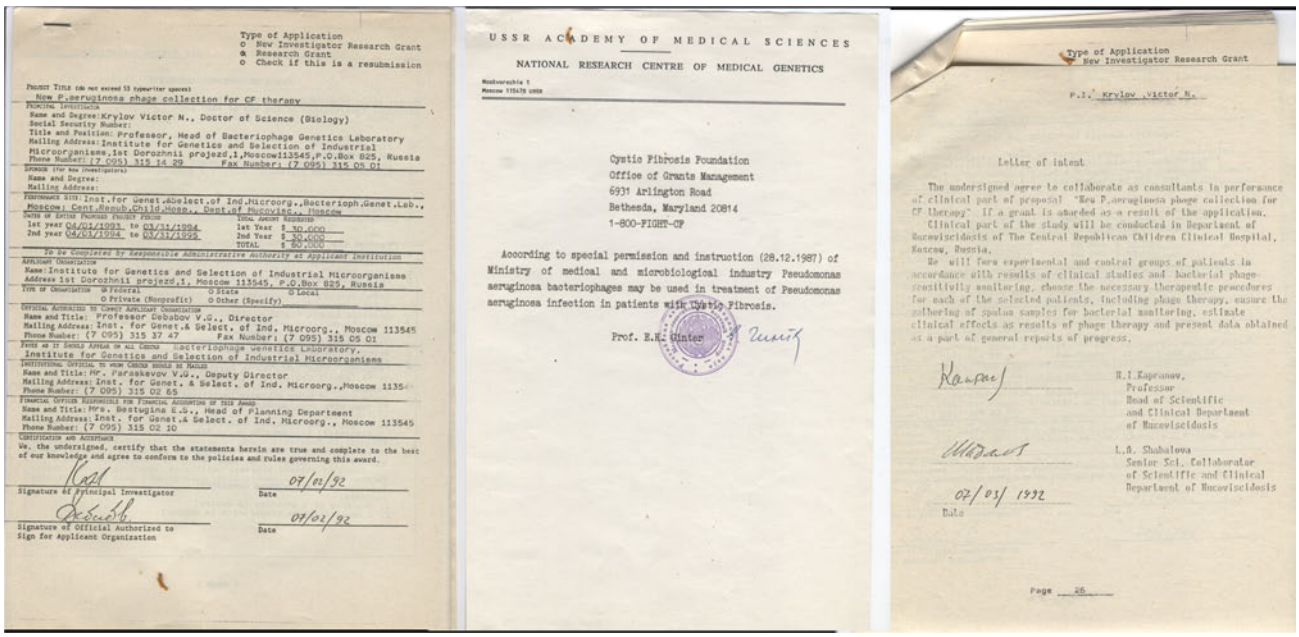
Critical Analysis of the Results of Phage Therapy in *Pseudomonas aeruginosa* Infections for a Group of Children With Cystic Fibrosis

The predominant goal of antibacterial therapy in CF is to cure chronic bacterial infections, especially those due to *P. aeruginosa,* with the aim of increasing patient life expectancy. This is of paramount importance in children up to 6 years of age, due to the inability to utilize colistin in extreme cases of multidrug resistant infection. Phage therapy in CF therefore should be implemented with this group in the forefront of our minds. The significance of the ongoing research will hopefully result in a resurgence of interest in phage therapy in the Western world, especially to support children suffering from CF.

Of relevance is collaborative research that took place in 1991–1992 between the Laboratory for bacteriophage genetics (being at that time in the Institute for Genetics and Selection of Industrial Microorganisms, Moscow, Russia), and the Department of Mucoviscidosis (CF) in the Children's Republican Clinical Hospital in Moscow. Following the acquisition of parental consent and permission from the Academy of Medical Sciences (**Figure 1**), a study was conducted into the use of *P. aeruginosa* phages in the treatment of five children with CF. Whilst there were no specific restrictions for the number of children to be involved in this research, the need to limit the number of participants was felt as being necessary due to the fact that this was the first trial involving the inhalation of phages for the treatment of CF associated infection and prudence is always wise in such uncharted territory. In addition, the phage preparations used were tailored and prepared in highly concentrated forms with purification on cesium chloride density gradients for participants to use over 7–10 days. Due to these rigorous protocols, laboratory resources were limited to a small number of patients.

The five children were aged from 7 to 12 years old. The compositions of the *Pseudomonas* strains in their lungs were studied. The differentiation of strains was performed by taking into account features such as resistance to clinically used antibiotics, mucus (alginate) production, colony pigmentation, temperate phage production, and sensitivity to various bacteriophages (including temperate) available at that time in the laboratory collection. Usually at least two different *P. aeruginosa* strains were found in the sputum of each child. The different mixtures of virulent bacteriophages were prepared for each child based on testing bacterial strain sensitivity to phages. The phages utilized were chosen among several species classified at the time, including phiKZ-, Lin68-, and PB1-like phages, and those of a group referred to later as the phiKMV-like phages. The phages were selected for each child based on the greatest lytic effect on strains isolated from sputum (those which resulted in transparent lysis in the spot assay). Selected phages were grown overnight in petri dishes with *P. aeruginosa* PAO1 by confluent lysis, followed by resuspension into saline and bacterial debris removed by centrifugation. The supernatant was treated with chloroform to eliminate residual bacterial cells and phages were further concentrated by polyethylene glycol precipitation. The resulting phage were then subjected to centrifugation in a cesium chloride gradient. Following dialysis, individual preparations were mixed with titers of at least 10^{11}–10^{12} particles/ml. Such high titres were used based on the assumption that a higher concentration of phages in inhaled mixtures would allow the determination of an effect (or lack of) in a short period of time. However, during the actual clinical application of the phage mixtures the preparations were diluted several times as it was found that concentrated mixtures were unable to pass through the inhalation device. The inhalations were carried out over the course of a week along with the standard monitoring protocols of the children's status.

It was found that one child had gone through a short period involving a transient increase in body temperature. The others had no obvious changes in their general condition. In two cases, comparison of bacteria in sputum samples after the course of phage therapy revealed evident changes attributable to the use of phages. In one of the sputum samples, were prevailing phage-resistant variants displaying the appearance exhibited by the original pigmented bacteria and the typical mucoid properties observed in *P. aeruginosa*. In the sputum of the second child



Type of Application
o New Investigator Research Grant
o Research Grant
o Check if this is a resubmission

New P.aeruginosa phage collection for CF therapy

Name and Degree: Krylov Victor N., Doctor of Science (Biology)
Social Security Number:
Title and Position: Professor, Head of Bacteriophage Genetics Laboratory
Mailing Address: Institute for Genetics and Selection of Industrial Microorganisms, 1st Dorozhnii projezd,1,Moscow113545,P.O.Box 825, Russia
Phone Number: (7 095) 315 14 29 Fax Number: (7 095) 315 05 01

Name and Degree:
Mailing Address:

Moscow; Cent.Konsult.Child.Hosp., Dept.of Microbiol., Moscow

1st year 04/01/1993 to 03/31/1994 1st Year $ 30,000
2nd year 04/01/1994 to 03/31/1995 2nd Year $ 30,000
TOTAL $ 60,000

To Be Completed By Responsible Administrative Authority at Applicant Institution

Name: Institute for Genetics and Selection of Industrial Microorganisms
Address 1st Dorozhnii projezd,1, Moscow 113545, P.O.Box 825, Russia

o Federal o State o Local
o Private (Nonprofit) o Other (Specify)

Name and Title: Professor Debabov V.G., Director
Mailing Address: Inst. for Genet.& Select. of Ind. Microorg., Moscow 113545
Phone Number: (7 095) 315 37 47 Fax Number: (7 095) 315 05 01

Institute for Genetics and Selection of Industrial Microorganisms

Name and Title: Mr. Parnskevov V.G., Deputy Director
Mailing Address: Inst. for Genet. & Select. of Ind. Microorg.,Moscow 1135
Phone Number: (7 095) 315 02 65

Name and Title: Mrs. Bestugina E.S., Head of Planning Department
Mailing Address: Inst. for Genet.& Select. of Ind. Microorg., Moscow 113545
Phone Number: (7 095) 315 02 10

We, the undersigned, certify that the statements herein are true and complete to the best of our knowledge and agree to conform to the policies and rules governing this award.

Signature of Principal Investigator — Date 07/01/92

Signature of Official Authorized to Sign for Applicant Organization — Date 07/02/92

USSR ACADEMY OF MEDICAL SCIENCES

NATIONAL RESEARCH CENTRE OF MEDICAL GENETICS

Cystic Fibrosis Foundation
Office of Grants Management
6931 Arlington Road
Betheeda, Maryland 20814
1-800-FIGHT-CF

According to special permission and instruction (28.12.1987) of Ministry of medical and microbiological industry Pseudomonas aeruginosa bacteriophages may be used in treatment of Pseudomonas aeruginosa infection in patients with Cystic Fibrosis.

Prof. E.K. Ginter

Type of Application
New Investigator Research Grant

P.I. Krylov ,Victor N.

Letter of intent

The undersigned agree to collaborate as consultants in performance of clinical part of proposal "New P.aeruginosa phage collection for CF therapy" if a grant is awarded as a result of the application.
Clinical part of the study will be conducted in Department of Mucoviscidosis of The Central Republican Children Clinical Hospital, Moscow, Russia.
We will form experimental and control groups of patients in accordance with results of clinical studies and bacterial phage-sensitivity monitoring, choose the necessary therapeutic procedures for each of the selected patients, including phage therapy, ensure the gathering of sputum samples for bacterial monitoring, estimate clinical effects as results of phage therapy and present data obtained as a part of general reports of progress.

N.I.Kapranov, Professor Head of Scientific and Clinical Department of Mucoviscidosis

L.A. Shabalova Senior Sci. Collaborator of Scientific and Clinical Department of Mucoviscidosis

07/03/ 1992
Date

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FIGURE 1 | Official permission for application of phage therapy in CF unit of The Central Republican Children Clinical Hospital, Moscow, Russia and agreement of physicians in CF unit to collaborate in the study (for request of grant support).

were phage-resistant variants of *P. aeruginosa* exhibiting novel properties. It was proposed that in this second case the original phage sensitive bacterial cells were substituted for another phage-resistant strain, with the new properties, possibly due to cross-infection from other patients.

The emergence of phage resistant variants of the initial infecting bacterial strain in one child and the substitution of sensitive bacteria for a new phage resistant strain in the lungs of the second child is clear evidence as to an effect from phage therapy *in vivo*. The absence of detectable changes in three other children could be attributed to various reasons such as too low a concentration of phage mixture following dilution of the original formulation, the high viscosity of mucus hindering the spread of the phage, and insufficient duration of phage application. These results were presented in several CF meetings and were considered as initial evidence for the safe use of phage therapy in CF (Shabalova et al., 1993, 1995). Unfortunately derivative studies were unable to be carried out at the time due to insufficient funding opportunities. However, giving the growing antibiotic resistance, this is starting to change. The use of commercial preparations of phages in the treatment of *P. aeruginosa* infection in children with CF in Georgia also has shown some promise (Kutateladze and Adamia, 2010).

THE MODULAR COMPOSITION PRINCIPLE FOR THE PREPARATION OF THERAPEUTIC PHAGE COCKTAILS

The pediatric study described above showed that personalized phage preparations can have a noticeable effect on a brief timescale. The previously active *P. aeruginosa* strains in lungs of two children were eliminated and were substituted with other strains. Independent of their origin (resistant mutant or cross infection), such situations require immediate re-evaluation of the therapeutic mixture for each of the two children showing positive reactions. The use of commercial preparations containing uncharacterized isolates is not an appropriate course of action. Therefore, a series of rapid protocols needs to be set in place to permit the rapid isolation, characterization and choice of phages and to allow the appropriate modification of preparations within a therapeutic timeframe. All of this must to be carried out in a safe and pragmatic manner.

We propose a method to enhance the rate at which phage mixtures are prepared for personalized therapy in CF through an alternative approach that will also permit the enhancement of the lytic spectra of the resulting preparation. The fundamental idea is to utilize what we refer to here as a modular principle. These modules may be monospecies phage mixtures or a mixture of phages of unrelated species taken from a collection of pre-selected phages. The advantage of this approach is that the expansion of phage activity of such mixtures has not been achieved through a one-time introduction of unknown phages in the final product, but introduction into each such modular product, a new phage of the same species but with enlarged activity for monospecies mixtures or some another new phage into a heterospecies mixture. In a joint multiplication of such mixtures, closely related phages may recombine, potentially resulting in progeny exhibiting novel spectra of lytic activity.

Thus, to transition to this novel approach we propose (1) to select a limited number of well-studied phage groups, containing a great deal of described non-identical phages and compose

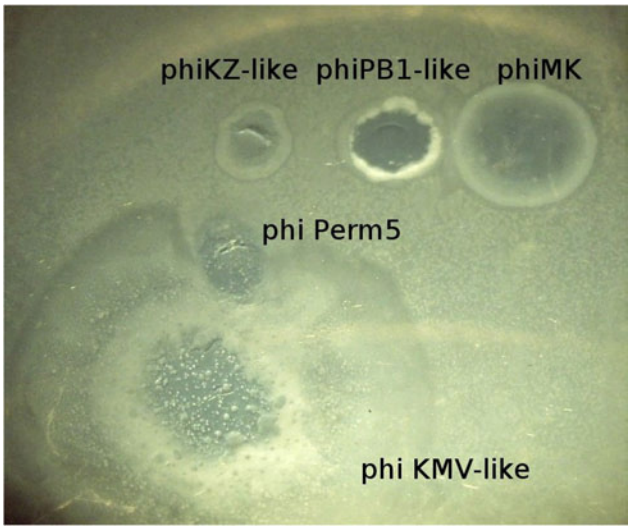

FIGURE 2 | Different growth appearance on *P. aeruginosa* PAO1 lawn permits preliminary identification of five bacteriophage species.

from these phages monospecies mixtures displaying unique lytic spectra; (2) merge, into a common single multispecies blend, well studied bacteriophages of other known species, with unique spectra of lytic activity; (3) Each of the preparations are propagated through infection of *P. aeruginosa* PAO1 or other acceptable strain; (4) The final therapeutic preparation for personalized therapy is composed of individual mixtures (modules) to allow for maximal lytic activity.

Obviously, the extensive accumulation and study of phages for each of the chosen species exhibiting a wide spectrum of lytic activity is an absolute prerequisite for the operation of this protocol. As described above, even short-term use of mixtures of constant composition from a small number of phages leads to the selection of resistant bacteria. Up until now there are only individual studies on the frequency of phages capable of infecting different bacterial species. For example, there is a report describing phages capable of infecting the bacterial species *P. aeruginosa* and *Burkholderia cepacia* (Nzula et al., 2000). But it is important to consider and evaluate potential interspecies transduction in such instances. In another study (Malki et al., 2015), the authors demonstrate that PB1-like phages of *P. aeruginosa* isolated from a natural habitat can infect unrelated species of the *Arthrobacter, Chryseobacterium,* and *Microbacterium* genera. It also suggests the possibility of interspecies transduction with potentially unpredictable results. Of course, despite this possibility, this is a natural inevitability associated with bacteriophages, and is independent of the phage therapy process (Penadés et al., 2015). Complete prevention is therefore not a realistic goal. However, we believe that additional studies are necessary to gain an insight into the effect of horizontal gene transfer with *in vivo* applications of phages.

Of critical significance in the selection of phage species for treatment of pseudomonade infection in CF is the ability to infect different natural *P. aeruginosa* strains. From a practical standpoint this is achieved through the recognition of such susceptible strains through the appearance of plaques. Moreover, in primary pulmonary infections of patients with CF, any strain of *P. aeruginosa* is capable of participation. As a general dogma, in the process of producing future proof therapeutic mixtures, the best species of phages form the foundation upon which refresh cycles take place using new modules to expand the overall lytic activity as necessary. Based on these general considerations and prior experience, we have chosen three phage groups suitable for composing integral monospecies phage mixtures as a foundation: phiKZ-like, phiKMV-like, and PB1-like phages. Their frequent occurrence in current commercial mixtures may reflect the broad spectrum of their lytic activity. In addition, they are well characterized – many genomes of phages in these groups have been sequenced and annotated. Finally, phages of these species produce recognizable plaques (**Figure 2**) making it easy to identify them amongst mixed populations in natural samples. There are, however, other phage species which seem promising for additional monospecies phage mixtures and which form the basis for subsequent research.

FEATURES OF SPECIES SELECTED FOR MONOSPECIES PHAGE MIXTURES AND CHOOSING THE BEST SOURCES OF THESE PHAGES

Table 1 lists the general characteristics of a group of selected virulent phage species active on *P. aeruginosa* (Adams et al., 2015). The function of most gene products of these phages remains unknown. Therefore, the choice of these phage species for therapy is based mainly upon the results of long-term work in several laboratories and, in addition, on the frequent presence of these phages in commercial therapeutic mixtures, from the days of D'Herelle. It is possible to assess the efficiency of different phages by determining their proportionality following a therapeutic application using plaque phenotype and PCR and then appropriately adapt the mixture for the next round of phage therapy. Below is an overview of the major groups of phages proposed to be utilized within monospecies and heterospecies mixtures.

The phiKZ-Like Viruses

The phiKZ-like group of giant phages infecting *P. aeruginosa* includes several species and are of interest not only because of their use in a therapeutic setting, but also as a unique model for the study of phage evolution and specific packaging of the genomic DNA (Burkal'tseva et al., 2002; Mesyanzhinov et al., 2002; Krylov et al., 2003, 2004, 2005, 2011; Hertveldt et al., 2005; Shaburova et al., 2008; Pleteneva et al., 2010; Sokolova et al., 2014). This group of *Myoviridae* phages includes several phage species that exhibit common properties of the type member phiKZ (particle size and morphology, the presence of an inner body in the capsid, and a specific packaging mechanism of phage genomic DNA). In sequenced and annotated genomes of two different species of this group – phiKZ and phiEL, genes encoding

TABLE 1 | *Pseudomonas aeruginosa* phage species, proposed for use in phage compositions as mono- or heterospecies mixes.

No	Subfamily and/or genera	Phage family	Representatives of phage species in our collection (No in NCBI)	The number of phages in the database NCBI on April 2016	Genome size Min – Max bp	The number of ORFs	The number of ORFs with known function	Our collection
1	Phikmvlike viruses	Podoviridae	phiKMV (NC_005045)	14	42351 – 43639	47–56	Max 28	phiKMV, phiNFS (+8 phages)
2	Pbunalike virus	Myoviridae	phiPB1 (NC_011810)	29	64144 – 68871	85–97	Max 33	phiPB1, F8, SN, 14/1 (+9 phages)
3	Phikzlike viruses	Myoviridae	phiKZ (NC_004629)	2	266743-280334	306–333	Max 144	PhiKZ (+17 phages)
4	Luz24like viruses	Podoviridae	phiTL (NC_023583)	10	44030 – 45808	58–73	Max 28	phiTL, phiCHU
5	Phikzlike viruses	Myoviridae	phiEL (NC_007623)	1	211215	201	21	phiEL, phiCHE, phiRU
6	Felixounavirinae PAKP1like virus	Myoviridae	phi MK (KU761955.1)	25	83598 –94555	149–188	Max: 28	phiMK
7	N4like virus	Podoviridae	PhiPerm5*	13	72028 –74901	83–115	Max: 26	phiPerm5
8	Phikzlike viruses	Myoviridae	phiLin68*	N/A	N/A	N/A	N/A	phiLin68, phiLBG22

*Genome is not sequenced.

a DNA polymerase have not been found. This may prove to be a common feature for this group. The genomes of PhiEL and phiKZ differ in their sizes and lack any tangible homology at the nucleotide level. Two other phages closely related with phiEL are phiRU (isolated from soil) and phiCHE (from a clinical strain of *P. aeruginosa* isolated from an infected burn wound). phiEL-like phages may be crossed in different combinations, giving rise to viable progeny. This is potentially useful for generating recombinants displaying variable host specificities both *in vitro* and *in vivo*.

Phages phiLin68 (of Lindberg phage collection) and phiLBG22 (our lab collection) are representatives of the third group in the phiKZ-like viruses. These phages were included due to the fact that they can lyse some strains resistant to phages from the phiKZ and phiEL groups. We have found non-identical phiKZ-like bacteriophages in all therapeutic commercial mixtures from different manufacturers. Interestingly, phiEL- and phiLin68-like phages have not yet been found within therapeutic mixtures. In creating therapeutic preparations containing phiKZ-like phages, one should bear in mind that all species in this group have the ability to transfer bacterial cells into persisting ("pseudolysogenic") state (Pleteneva et al., 2010). It has similarity with effect of lysis inhibition after infection of *E. coli* bacterial cells with wild type phage T4 but not with r-type mutants (rapid lysis) (Doermann and Hill, 1953). A possible reason may be the maintenance of certain structures in cell envelope after the re-infection of bacteria with wild-type phage (Krylov, 1970). Meanwhile phiCHE bacteriophage, closely related with phiEL, has been isolated from a bacterial strain in burn wound. Different mutations in genomes of phiEL-like phages influence the effect (Krylov et al., 2011). This is manifested through the appearance of opalescence of bacterial growth (**Figure 3**) and a significant increase in the final yield of phage. In this state, however, the lysis of infected bacteria is delayed. For this reason, it is not desirable to use wild type phiKZ-like phages as components in therapeutic preparations for the treatment of *P. aeruginosa* pneumonia. In addition, amongst *P. aeruginosa* strains isolated from the lungs of CF patients are often those strains capable of producing autoplaques (Pillich et al., 1985). We have found that *in vitro* infection of clinical isolates in CF with different wild type phiKZ-like phages leads to a decrease in the level of autoplaquing of these bacteria. As a result, the growth of phiKZ-like pseudolysogenic bacteria occurs more rapidly than the growth of the original uninfected bacteria (**Figure 4**). Therefore, to maintain the possibility of using the wide lytic potential of phiKZ-like phages in CF in a safe manner, we have isolated a group of mutants in phages phiKZ and phiEL which have lost the ability to transfer bacteria into the pseudolysogenic state (Krylov et al., 2011). Some of these mutants are also showing signs of hypervirulence, capable of lysing pseudolysogenic bacteria. We believe that mutant phages such as these, belonging to the phiKZ- like group can be used safely as part of the therapeutic mixtures, complementing each other's activity and thus providing us with an additional weapon in our arsenal.

The decision to include phages of Lin68 species into a therapeutic mixture could be made after the isolation of similar

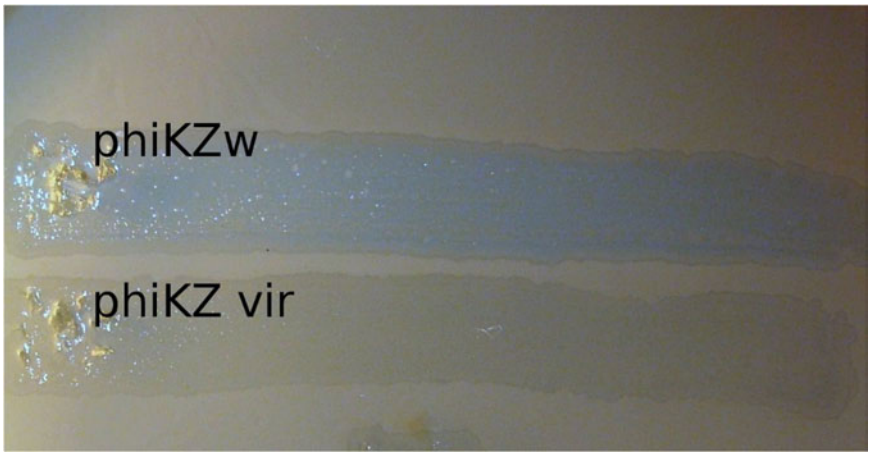

FIGURE 3 | The vir-mutant of bacteriophage phiKZ is unable to enter the pseudolysogenic state.

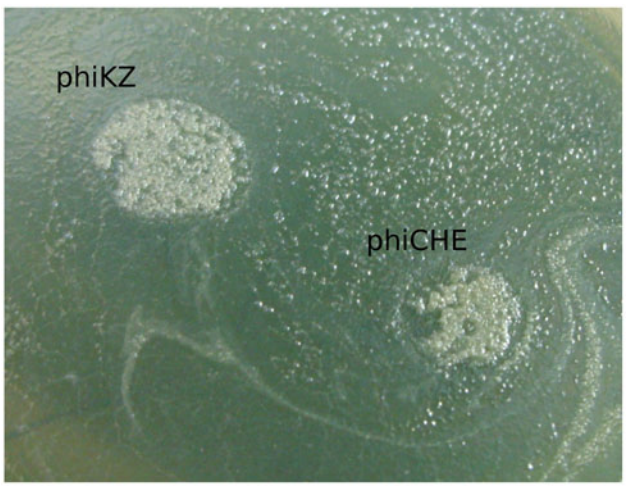

FIGURE 4 | The growth of phiKZ and phiCHE pseudolysogens in places of phages application on the background of auto-lysed lawn of plaque forming clinical isolate *P. aeruginosa* CF017 after prolonged incubation.

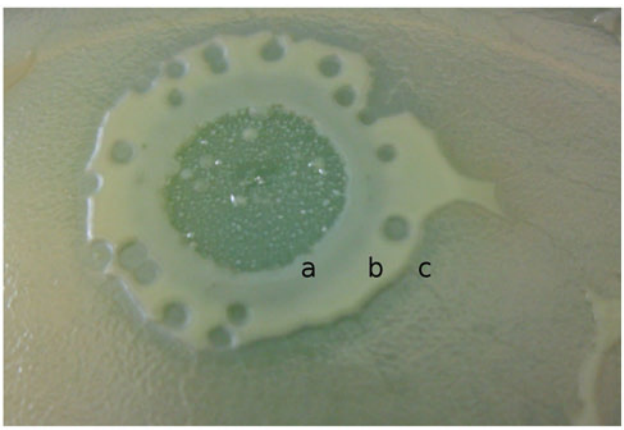

FIGURE 5 | The growth of KMV-like phage phiNFS on lawn of plaquing *P. aeruginosa* clinical isolate inhibits plaque formation. The different activities of diffusible products can be observed: **(a)** complete inhibition of plaquing; **(b)** a significant reduction in the size of the plaques to a certain size; **(c)** an abrupt discontinuation of interaction. This is an example of a phage–host interaction which can seriously influence the results of phage therapy by enhancing bacterial growth in lung biofilm. Only specially prepared phage mixture can prevent undesirable effects of mono phage therapy.

mutants and (preferably) after the sequencing and annotation of their genomes, as done for phiKZ and phiEL.

PhiKMV-Like Bacteriophages

phiKMV – related bacteriophages (Lavigne et al., 2003, 2006; Burkal'tseva et al., 2006) are also frequent components of the various commercial mixtures and display different host ranges and plaque sizes to that of other groups. For example, a new phiKMV-like phage, phiNFS, has been isolated as a mutant from a commercial mixture by our group. The parental phage forms small transparent plaques on the lawn of strain PAO1 and in each such plaque mutants with increased growth rate have arose at high frequency (Krylov et al., 2015). The possible reason for this effect may be related with the use of a bacterial host exhibiting properties that differ from those in *P. aeruginosa* PAO1 in the production of the commercial mixture. Mutants isolated from various plaques may vary in growth rate on different clinical isolates. The ability for permanent modifications of different growth characteristics is an inherent feature of the phage phiNFS. The mechanisms underpinning this are under investigation. Preliminary data suggest that it may be related to processes involved in quorum sensing. For example, in some clinical isolates phage phiNFS specifically inhibits the formation of bacterial plaques with a clear border of inhibition observed (**Figure 5**), which can be explained with the production in plaque growth of an unknown product which interacts specifically with cells of the plaque producing bacterial strain ("all or nothing"). This phage displays the greatest expression of a peculiar feature of

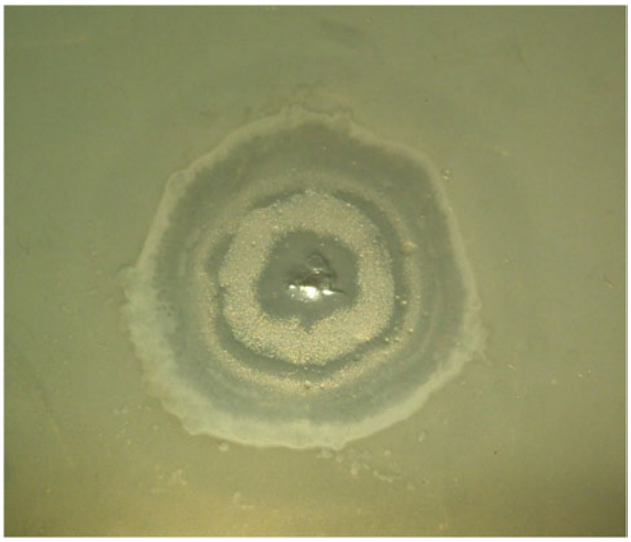

FIGURE 6 | The continuing phiKMV-like phage growth going through cyclic periods of lytic growth and pseudolysogeny.

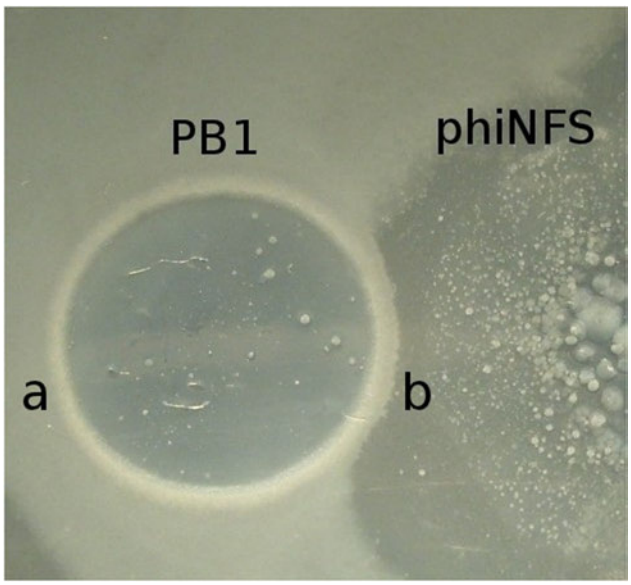

FIGURE 7 | The common feature of all PB1-like phages are specific bacterial bands bordering their plaques. Such bands contain special bacterial cells in the pseudolysogenic state and which limit the growth of phages of other species. In some cases this property can influence the result of phage therapy **(a)** The growth of cells in pseudolysogenic state around phiPB1 phage plaque. **(b)** Interaction of lytic areas of bacteriophages phiPB1 and phiNFS.

the phiKMV-like phages: continuous (5–7 days) growth on aged bacterial lawns, something uncharacteristic of other species of *P. aeruginosa* phages. The cyclic nature of this growth may be due to the ability of the phage to overcome the physiological changes in bacteria arising as result of the aging process (**Figure 6**).

PB1-Like Bacteriophages

PB1-like bacteriophages are another frequent representative of phage cocktails. A clear plaque with a characteristic feature - a dark stripe along the edge of the growth (**Figure 7a**) is a typical feature of the group and may be used as a species characteristic in looking for new phages of the group. This stripe appears at the end of plaque growth and it consists of infected cells in a specific state (Krylov et al., 2015). It limits the spread of the plaques of other phage species (**Figure 7b**) but is unable to prevent penetration of phage particles into PB1 plaques. Advantages of the PB1-like phages are in the simplicity of their selection from enrichment of therapeutic mixtures (non-identical phages can be often be found in samples taken from the same natural water sources), a wide range of lytic activity, and a relatively low frequency of phage resistant mutant generation (Garbe et al., 2010).

Heterospecies Phage Cocktail

Besides the three monospecies mixtures, we suggest the use of an additional mixture comprising phages of several unrelated species. Each of these phages display certain differences in their spectrum of lytic activity, for example, the ability to lyse bacteria exhibiting resistance to phage mixtures of the three previously mentioned major species, as well as strains carrying a plasmids conferring phage-resistance. This group includes phages phiEL (NC_007623), phiLin68, phiPerm5, phiMK (KU61955), and phiCHU (NC_028933), representing several different groups. As all the phages of those selected for this mixture are relatively easy to recognize (Krylov et al., 2015) the choice of new phages for the purpose of expanding the lytic spectrum will face little in the way of problems. Nevertheless, it is of critical importance to pay special attention to the possibility of significant differences existing between closely related phages. Sometimes their genomes can contain small variations which result in them being inappropriate for therapeutic use. For example, phages phiTL and phiCHU showed good growth on PAO1 in the presence of IncP2 group plasmids (for example, pMG53 plasmid) and on some clinical isolates resistant to other phages. The improved growth of phages is accompanied with occurrence of unstable plaque morphology (**Figure 8**). However, only phiCHU can be utilized in therapeutic mixtures as the phiTL genome was found to encode a putative transposase fusion gene (NC_023583, Magill et al., 2015). These phages are closely related to LUZ24 (Ceyssens et al., 2008; Wagemans et al., 2015) and phiPaP3 (Tan et al., 2007), together belonging to the LUZ24likevirus genus.

P. aeruginosa PAO1: THE BEST HOST FOR THE STUDY OF NEW PHAGES, BUT NOT FOR THE MULTIPLICATION OF THERAPEUTIC MIXTURES?

In some cases, problems may arise when trying to find a suitable strain for the amplification of finalized therapeutic mixtures. One such problem is that *P. aeruginosa* strains often contain the prophages of filamentous phages. The

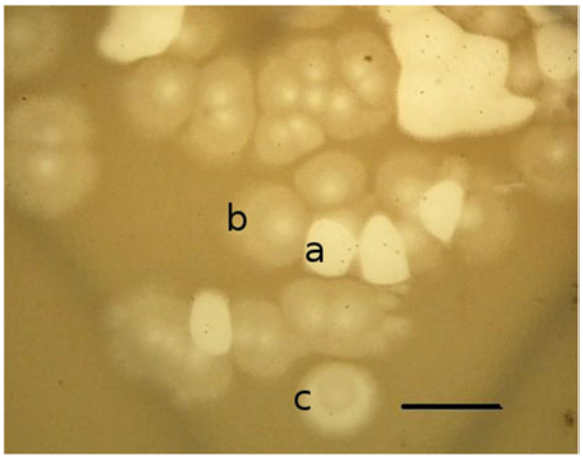

FIGURE 8 | Diversity of bacteriophage phiCHU plaques on lawn *P. aeruginosa* PAO1 (pMG53). The replanting of phages from clear **(a)**, turbid **(b)** or semiclear **(c)** plaques again leads to production of all types of plaques. Bar is 1 cm.

reason for the frequent occurrence of filamentous prophages is believed to be that they provide adaptive capabilities to *P. aeruginosa* under unfavorable conditions (Rice et al., 2009). The accumulation of filamentous phages within the mucus of the lungs promotes crystallization and establishment of solid biofilms (Secor et al., 2015). Therefore, choice of hosts containing these prophages (such as *P. aeruginosa* PAO1) can result in the unintentional inclusion of filamentous phages into the final preparation. The therapeutic use of a phage mixture obtained as a result of random enrichment and amplification on an unstudied host strain is therefore undesirable. In addition, one should note that despite the fact that the PAO1 strain of *P. aeruginosa* is a well studied and long standing work horse of many microbiology laboratories, one should be careful with respect to its potential use in the final amplification of phage preparations. This is due to the fact that it possesses a fairly high pathogenic potential, as seen by its long term presence within the lungs of mice, though it is advantageous for the prevention of introducing temperate phages with undesirable features (Tayabali et al., 2015). It does, however; remain as the accepted strain for the study of the phages themselves.

The work on the creation of a reserve collection of new phages for inclusion into monospecies phage mixtures should be carried out on an ongoing basis. In accordance with our experience, the selection of new phages and reproduction of monospecies mixtures would be better accomplished with the strict use of the standard strain of *P. aeruginosa* PAO1 and its minor variants (e.g., those carrying plasmids for propagation of plasmid dependent phages such as phiCHU). The use of the standard strain PAO1 is necessary for preventing the introduction of potential temperate phages with undesirable features (for example, transposable capabilities) into therapeutic phage mixtures. It is evident, joint multiplication of non-identical but closely related phages can and will produce valuable recombinants, including ones with an increased spectrum of lytic activity. Thus the simple act of reproduction of a monospecies mixture will create an additional opportunity to expand the lytic spectrum.

"NON-ESSENTIAL" GENES AND THE PROSPECT OF "CORRECTING" THE GENOMES OF BACTERIOPHAGES

We are confident that phage therapy of *P. aeruginosa* infection in children with CF is a very promising approach and is by no means limited to the use of natural phages from well-studied groups. It is likely that novel methods for genome editing will be utilized in the enhancement of therapeutic phages. This will permit less time consuming selection procedures for phages exhibiting novel lytic spectra. It may therefore be of some interest to investigate the reasons behind significant differences in genome sizes of phages exhibiting identical morphologies, due to the presence in the genomes of some phage species, regions which contain potentially non-essential genes with accessory functions. Such phages could be used as convenient vectors. For example, in phages phiKZ and phiEL which belong to different but phylogenetically related groups of giant phiKZ-like phages, we observe that phage particle sizes and the internal volume of the capsid are similar. The genome of phiEL, however, is ∼80 kb less than that of phiKZ. The cause of this difference may be, for example, a discrepancy in evolutionary rates of the morphological structure of the phage particle and its genome size. One must also consider that increases in genome size are limited by the capacity of the phage particle, so it could be reasoned that evolution would favor smaller genome sizes due to physical constraints. The viability of phage particles (in particular, the ability of DNA be injected into cell) though, may depend on the density of DNA packaging, and the loss of a significant number of genes, even those whose functions have ceased to be significant in the development of the phage, could prove lethal. In such cases there are two different strategies that may permit phage survival. One such strategy would be the evolution of a novel DNA packaging mechanism. Another possibility is via the conservation of genes whose functions become unnecessary or to replace them by similarly sized DNA sequences of temporal origin. As it happened, both of these strategies have been implemented. In the case of phage phiEL a variable packaging mechanism has arisen, which provides the necessary density for DNA packing and thus injection (Sokolova et al., 2014). Another postulated mechanism can be illustrated by comparing the genomes of two closely related phages of *P. aeruginosa* PAO1 – temperate phage phiD3 and its naturally lytic variant phiPMG1 (Krylov et al., 2012). In the central part of the genome of these phages there exists a large region containing gene fragments of variable origin. The presence of extensive areas of insignificant DNA ("genome gaps"), is an apparently common phenomenon. They were also found when comparing of the genomes of two transposable phages, PM105 and B3 (Pourcel et al., 2016).

Phages which possess these genome gaps could form a solid basis for the directed design of therapeutic phage genomes, for instance to insert genes that increase the efficiency of phage lysis. For example, the insertion of genes encoding bacteriocins, capable of killing bacteria of other pathogenic species, usually associated with CF. This idea is extremely promising but will require not only knowledge of the detailed functions of all genes and their interactions, but also evidence that the introduction of new genes will not result in adverse unwanted effects. Also, development and use of such future artificial phages will require thorough study of their potential interactions with the natural microbiota in humans and their potential environmental impacts.

Expanding the Collection of Therapeutic Phages: National and International Phage Bank Cooperation

The implementation of continuous personalized phage therapy will require a cooperative and efficient system to allow the exchange of specific bacteriophages. The existence of a central phage bank keeping most of the phages active on *P. aeruginosa* and other pathogens active in CF and ensuring their availability for clinical laboratories in different countries would become an extremely valuable resource in addition to national collections. The basic purposes of this central phage bank would be:

(1) The accumulation of *P. aeruginosa* phages, their classification and comparison of their specific activities;
(2) Storage of phages which have been certified as safe;
(3) Composing initial monospecies phage mixtures with wide host range to adapt mixtures for real time requirements in hospitals;
(4) The delivery to hospitals of small samples of different phages to help clinicians and laboratory staff carry out the rapid selection of appropriate phages.

The understanding for the necessity of forming such phage banks is now generally accepted. Such institutions will form the central hub from which phage therapy can be developed.

CONCLUSION

One of the purposes of this review was to show that choosing phages suitable for therapeutic use requires not only genomics and bioinformatics approaches, but long-term preliminary studies of their properties and their manifestations under different conditions in order to optimize their activity. The other purpose was to introduce a novel approach for the composition of therapeutic mixtures based upon a modular assembly of personalized preparations utilizing combinations of mono- and hetero- species mixtures to use in the treatment of *P. aeruginosa* infections in CF patients.

There is no doubt as to the efficiency of phage therapy when the correct selection of phage has been achieved. As discussed, this has been confirmed in experiments involving the eradication of *P. aeruginosa* lung infection in mice following intranasal administration of virulent phages (Alemayehu et al., 2012; Henry et al., 2013). There is also indirect confirmation of a positive response to phage activity under clinical conditions following from the observation (James et al., 2015) whereby authors found a correlation between increases in the number of free temperate phage particles in the sputum and improvements in the patient's condition. This is understandable, because infection of bacterial cells with temperate phages usually results in the lytic cycle being the predominant mode of phage development. However, this study does not support the deliberate use of temperate phages. Finally, an example of the direct use of personalized phage mixtures in clinical practice described here reinforces the overall safety of the procedure itself.

Thus, we believe that phage therapy in CF is promising, but its use should be limited to those special cases whereby all available antibiotics have been proven to be ineffective. The group of potential patients includes children up to 6 years of age infected with multi-resistant strains of *P. aeruginosa* (as the use of colistin is not yet permitted) or adult patients infected with *P. aeruginosa* resistant to all available antibiotics, including colistin. As a matter of fact the transition to the permanent use of phage therapy, even in special cases, will require a significant change in the workings of specialized clinics. There must be special measures in place to separate the patients under phage therapy from others so as to prevent cross infection with other *P. aeruginosa* strains. The best solution would be the organization of a special center of phage therapy for CF patients, providing necessary resources and the capability to support and enlarge phage collections, etc. The work of personnel in such centers will include procedures utilized on a daily basis in academic laboratories. The aim of specialized teams of microbiologists in hospitals working with phages will be to monitor the changes in the proportion of bacteria resistant to used phages, developing and scheduling the introduction of new phage preparations, creating new combinations of compounds that extend the lytic spectrum, all procedures that should ensure the continuity of phage therapy. We believe that if certified manufacturers of commercial phage mixtures were to participate in the preparation of proposed mono- and heterospecies phage mixtures, it would both simplify and greatly in diminish the response time in the preparation of personalized cocktails and thereby help phage therapy become established as a major weapon in our arsenal against bacterial infection.

AUTHOR CONTRIBUTIONS

VK: corresponding author, organization of the work, writing of the article. OS: preparation of pictures, editing of text. EP, OP, and EC: selection and testing of phages, composition of phage mixtures, data analyze. MB, SK, and AK: DNA analysis of phages different species. LK and DM: DNA sequence of new phages, analysis of data, editing and correction of text.

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REVIEW
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Prospects of Phage Application in the Treatment of Acne Caused by *Propionibacterium acnes*

Ewa Jończyk-Matysiak[1], Beata Weber-Dąbrowska[1,2], Maciej Żaczek[1], Ryszard Międzybrodzki[1,2,3], Sławomir Letkiewicz[2,4], Marzanna Łusiak-Szelchowska[1] and Andrzej Górski[1,2,3]*

[1] Bacteriophage Laboratory, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [2] Phage Therapy Unit, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [3] Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland, [4] Medical Sciences Institute, Katowice School of Economics, Katowice, Poland

Propionibacterium acnes is associated with purulent skin infections, and it poses a global problem for both patients and doctors. Acne vulgaris (acne) remains a problem due to its chronic character and difficulty of treatment, as well as its large impact on patients' quality of life. Due to the chronic course of the disease, treatment is long lasting, and often ineffective. Currently there are data regarding isolation of *P. acnes* phages, and there have been numerous studies on phage killing of *P. acnes*, but no data are available on phage application specifically in acne treatment. In this review, we have summarized the current knowledge on the phages active against *P. acnes* described so far and their potential application in the treatment of acne associated with *P. acnes*. The treatment of acne with phages may be important in order to reduce the overuse of antibiotics, which are currently the main acne treatment. However, more detailed studies are first needed to understand phage functioning in the skin microbiome and the possibility to use phages to combat *P. acnes*.

Keywords: *Propionibacterium acnes* phages, experimental phage therapy, treatment of infections, antibiotic resistance, topical application

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Peter Mullany,
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University of Guelph, Canada

****Correspondence:***
Ewa Jończyk-Matysiak
ewa.jonczyk@iitd.pan.wroc.pl

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INTRODUCTION

We are entering the post-antibiotic era: infections formerly easy to cure are becoming difficult to treat, as both increasing frequency of treatment failure and increasing severity of infections have been observed (Jassim and Limoges, 2014; WHO, 2014). The number of new approved antibiotics has dramatically decreased, and research on these new drugs is difficult, arduous, and unprofitable (Clarke, 2003; WHO, 2014).

At present, ∼50 million people in the US are suffering from acne, 85% of whom are at the age of 12–25 (Sidbury and Paller, 2000; Lynn et al., 2016). Disorders arising as a result of the reaction to acne can lead to a significant reduction in self-esteem (Sidbury and Paller, 2000). The inflammatory form of acne may leave scars that can result in permanent disfigurement. Correct and appropriate use of antibiotics in the treatment of acne will help to preserve their utility in the face of increasing antibiotic resistance, and therefore greater awareness of the issues is required among prescribing physicians (Dréno, 2016). The chief problem with antibiotic therapy of acne is the common tendency to prolonged use and overuse of antibiotics. Therefore, it is necessary

to treat acne with effective alternatives to commonly used antibiotics to reduce the likelihood of resistance to this type of treatment (Walsh et al., 2016) and obtain a highly specific agent to destroy bacteria effectively. The US National Institutes of Health (NIH, US) indicates phages to be innovative components that may be used to combat antibiotic resistance (NIH, 2014).

Detailed understanding and expanded knowledge of the microbiome, especially that of human skin, may be fundamental for recognizing skin-associated pathogenesis, especially acne vulgaris, and finding therapeutic solutions (Marinelli et al., 2012), e.g., the possibility to develop phage therapy of this disease. Further and more detailed studies are needed to understand phages' function in the skin microbiome and their participation in resistance gene transfer (Hannigan and Grice, 2013).

PATHOGENESIS AND EPIDEMIOLOGY OF ACNE

Acne vulgaris is the most common human skin disease (Valente Duarte de Sousa, 2014). Despite the implementation of new-generation antibiotics, the disease continues to have a social dimension. According to previous studies, the skin of teenagers suffering from acne vulgaris has up to 100-fold higher numbers of *Propionibacterium acnes* compared to healthy skin (Leyden et al., 1975). However, subsequent studies have failed to detect any significant discrepancies in the number of *P. acnes* between patients and controls. The observed levels of *P. acnes* were almost identical, but increased levels of *S. epidermidis* were observed (Bek-Thomsen et al., 2008). The presence of specific phylotypes of *P. acnes* is thought to be associated with the disease rather than the abundance of *P. acnes* (Tomida et al., 2013).

The etiology of acne is multifactorial, but probably the main agent associated with acne is the bacterium *P. acnes*. This pathogen is associated with acne, but the causality of the disease is not clear, and the exact role of *P. acnes* in acne is controversial. Acne is a chronic dermatosis that may cause lesions observed as papules or nodules (Zaenglein et al., 2016). The clinical form of acne depends on the interaction of the following factors: malfunctioning shedding of hair follicle cells, excessive sebum secretion, colonization of hair follicles by *P. acnes*, and individual factors depending on the host (e.g., the status of the immune system; Sidbury and Paller, 2000). Oversecreted sebum is accumulated under interconnected keratinocytes which clog the outlet of the sebaceous glands, thus creating ideal conditions for *P. acnes* growth. These bacteria trigger inflammatory infiltration, and the inflammation can spread to the dermis. A severe form of acne is acne inversa (also known as hidradenitis suppurativa), a chronic inflammatory disease which has the most significant impact on patients' quality of life among all assessed dermatological diseases (Wollina et al., 2013). It may be observed especially in patients with altered immunity with deficiency of antimicrobial protein secretion (Wolk et al., 2011). Pain is a major symptom of this form of acne. Treatment is by application of a drug, sometimes even combined with surgery (Wollina et al., 2013).

Propionibacterium is one of the main components of human skin microbiota of healthy adults (Human Microbiome Project Consortium, 2012; Hannigan and Grice, 2013). It predominates in sebaceous regions and is estimated to represent nearly 90% of the microbiota (Fitz-Gibbon et al., 2013). It is an opportunistic, Gram-positive, microaerophilic, nonmotile, and fat splitting bacteria, which—due to its tendency to elicit an inflammatory response—is thought to be the probable main cause of acne. The genomes of different *P. acnes* strains were found to be of similar sizes, G+C contents, and encode a similar number of open reading frames (ORFs) (Tomida et al., 2013). Another sequencing analysis suggested that acquired DNA sequences of *P. acnes* and its immune elements were important in determining the virulence of *P. acnes*, and these elements should be the therapeutic target (Fitz-Gibbon et al., 2013). According to Fitz-Gibbon et al. (2013), genomic comparison of *P. acnes* strains indicated that there may be specific genes that contribute to the pathology of acne. Holland et al. (2010) reported that *P. acnes* produces hydrolases likely to be involved in degrading human tissue components and immunoreactive adhesins which are expected to play a role in its virulence. Moreover, factors that have only been suggested to play a part in *P. acnes* pathogenesis (there is no experimental evidence) are: co-hemolytic CAMP factor 5, gehA lipase, putative hemolysin tly, sialidases, neuraminidases, endoglycoceramidases (Brüggemann, 2005). The facts that *P. acnes* may persist inside macrophages (prostate-infiltrating macrophages and the human macrophage cell line THP-1) and that it survives phagocytosis are very important for the pathogenesis of other diseases associated with this pathogen. This phenomenon may be important for *P. acnes*-associated inflammatory diseases (Fischer et al., 2013).

CONVENTIONAL TREATMENT OF ACNE

Antibiotics (both oral and topical) have been used as therapeutics for acne treatment for 40 years. It is estimated that every year ∼5 million prescriptions of antibiotics for oral treatment of acne are prescribed (Stern, 2000). In recent years, topical, enteral, and parenteral antibiotics have been used. The most commonly used are: nadifloxacin, ofloxacin, erythromycin, clindamycin hydrochloride, doxycycline, tetracycline hydrochloride, minocycline, ampicillin, cephalexin, gentamycin, and trimethoprim-sulfamethoxazole (Nishijima et al., 1996; Michałek et al., 2015). Currently, experimental trials are underway on a new generation quinolone—ozenoxacin *in vitro* (Choudhury et al., 2011). This quinolone is mainly for topical administration which is used as effective treatment for complicated skin and soft-tissue infections. Its mechanism is based on simultaneous affinity for two enzymes: DNA gyrase and topoisomerase IV (Karpiuk and Tyski, 2013). Due to the chronic course of acne vulgaris, treatment is long and often ineffective. Ozenoxacin application leads to shortened time of treatment. For the same reason, oral antibiotic therapy is supported by drugs applied topically, e.g., doxycycline (100 mg), which is administered orally once daily for 12 weeks and combined additionally with 5% dapsone applied topically twice

a day (Kircik, 2016). Nagler et al. (2016) reported that the average duration of antibiotic therapy in acne in some cases may even exceed 1 year (Nagler et al., 2016). Such prolonged therapy can be shortened by the addition of isotretinoin (a 13-cis-retinoic acid which is a non-aromatic retinoid; Tilles, 2014), whose action is associated with reduction of sebum production, anti-inflammatory properties, reduction in *P. acnes* and an effect on comedogenesis (decreasing hyperkeratinization; Layton, 2009). Isotretinoin is used to treat severe and recalcitrant cases of inflammatory acne. This drug significantly improves the results of acne treatment, but its application is associated with such adverse side effects as the ability to cause mental disorders, e.g., depression or suicidal thoughts, and even incidents of suicide have been reported. Nevertheless, improvement in mood and an increase in the quality of patients' lives after its application have been observed (Schrom et al., 2016).

Since prolonged antibiotic therapy in acne treatment is common, antibiotic resistance of *P. acnes* strains has been extensively observed (Sardana et al., 2016; Walsh et al., 2016). In 1976 there were no reports of antibiotic resistance in *Propionibacterium* strains (Leyden, 1976). But already 3 years later bacterial strains isolated from skin of patients with acne proved to be cross-resistant to erythromycin and clindamycin in both *in vitro* and *in vivo* studies (Crawford et al., 1979). Scientists and physicians have raised the alarm that an alternative to topical antibiotics should be found (Dréno, 2016). They suggest limiting the use of antibiotics, and especially avoiding their use as monotherapy (Zaenglein et al., 2016). It has been demonstrated that prolonged use of antibiotics generates selective pressure to induce antibiotic-resistant bacteria, and it correlates with the duration of these drugs' usage. Antibiotic resistance in these bacteria develops by spontaneous mutation rather than the horizontal transfer of multiple drug resistance genetic determinants (Moore and Sauer, 2008; Neely et al., 2008).

Using antibiotics may be associated with disturbance in natural microbiota, and it may cause the risk of colonization of such stains as *Streptococcus pyogenes* (Levy et al., 2003). Another danger that may result from application of antibiotics is the possibility of *P. acnes* to form an antibiotic-resistant biofilm. This natural state of bacterial colonization may protect bacteria against antimicrobials and facilitate bacterial adherence to tissue (Coenye et al., 2008; Portillo et al., 2013; Dréno, 2016). *P. acnes* has been shown visually to exist as biofilms on the skin by Jahns and Alexeyev (2014), who observed biofilm spreading for 1900 μm in a terminal hair follicle. To obtain the optimal absorption of e.g., tetracycline, it is recommended to be used on an empty stomach (Sidbury and Paller, 2000). It may cause an increase in the risk of occurrence of such adverse effects as gastrointestinal disorders. Moreover, in the case of women, candidiasis may develop. Also, acne vulgaris is thought to have an influence on the incidence of suicidal ideation in acne patients (∼7.1%; Kumar et al., 2016).

For the topical treatment of acne, antibiotic-containing creams, gels, solutions, and microemulsions are used (Sidbury and Paller, 2000).

BACTERIOPHAGES

Bacteriophages are bacterial viruses that naturally control microbial populations. They can multiply only in bacterial cells, and therefore may be active at the site of infection, where pathogenic bacteria are present. It is estimated that in the biosphere bacteriophages are 10 times more frequent than bacterial cells (Abedon, 2011). They are commonly found in the biosphere (Lin et al., 2010; Zhan et al., 2015) and human and animal organisms (Keller and Traub, 1974; Caroli et al., 1980; Gantzer et al., 2002; Bachrach et al., 2003; Reyes et al., 2012). Bacteriophage morphology shows great diversity, and their classification was traditionally based on the shape of the phage particle and the type of nucleic acid. However, the similarity in structure does not determine the biological properties of bacteriophages. Nowadays the phage classification is mainly based on DNA sequence identity (Kropiński et al., 2016).

Phages are called "living drugs" (Jassim and Limoges, 2014), and this term reflects the behavior of phages at the infection site. There are data confirming their efficacy in treating local and systemic infections caused by antibiotic-resistant bacterial strains (including those where bacteria resistant to multiple antibiotics are etiological agents; Biswas et al., 2002; Keen, 2012; Międzybrodzki et al., 2012; Chhibber et al., 2013; Borysowski et al., 2014; Rose et al., 2014; Cao et al., 2015; Sarker et al., 2016).

Phages have features that give them advantages over antibiotics, e.g., they are specific to their bacterial host (Ly-Chatain, 2014), which may minimize the probability of appearance of secondary infection (Golkar et al., 2014), and they multiply at the site of infection where there are sensitive bacteria (Loc-Carrillo and Abedon, 2011). The development of resistance of bacteria to antibiotics does not equate to simultaneous development of phage resistance in bacteria, although bacterial resistance to phages may also be observed. However, phage-resistant mutants that had lost phage receptors on the cell surface proved to be less pathogenic than phage-susceptible ones (Capparelli et al., 2010; León and Bastías, 2015). Phages are commonly found in the biosphere and therefore are environmentally friendly, because they are natural structures based on natural selection (Golkar et al., 2014). The isolation of new phages for therapeutic purposes is an affordable and rapid process compared to research and development of new antibiotics (which takes several years and may cost millions of dollars for clinical trials). Moreover, phage therapy is less expensive than conventional antibiotic therapy, which has been demonstrated in the case of patients with staphylococcal infections (Międzybrodzki et al., 2007).

Phages are proved to be safe and well tolerated, with few side-effects and without any toxic effects (Bruttin and Brüssow, 2005; Borysowski and Górski, 2008; Denou et al., 2009; Międzybrodzki et al., 2012; Miernikiewicz et al., 2013; Łusiak-Szelachowska et al., 2014).

An important aspect from the therapeutic point of view is the ability of phages to be temperate (e.g., co-exist with their host, either in a lysogenic, or pseudolysogenic state), as well as their ability to induce transduction. These processes may result in gene transfer between bacterial strains, as well as

antibiotic resistance genes or pathogenicity genes. These factors are critical for the safety of phage therapy. However, Modi et al. (2013) based on their research on antibiotic treatment of mice (ciprofloxacin or ampicillin) suggested that antibiotic application increased the frequency of phage integration into the bacterial genome, and enriched the phage metagenome for stress and niche specific functions, which shaped the phage-bacterial network to potentiate accessibility of phage genetic elements. Moreoever, the phage metagenome from mice treated with antibiotics was enriched with additional functions that may contribute to metabolism of the host (e.g., ampicillin-treated mice acquired a broader carbohydrate metabolic pathway).

To take advantage of the maximal phage potential, a regulatory framework should be established (Jassim and Limoges, 2014). Successful phage therapy requires phages of only proven lytic activity, whose lifecycle results from the burst size (Mirzaei and Nilsson, 2015). As mentioned above, especially personalized phage therapy (tailored therapy) that uses phages adjusted to specific pathogenic bacteria that are the cause of infection may be successful (Jassim and Limoges, 2014; Mattila et al., 2015) due to higher (5–6-fold) success rates (Zhukov-Verezhnikov et al., 1978) compared to *prêt-à-porter* preparations. It is proposed to develop improved methods of administration and new formulations that reduce the exposure of phage to destructive conditions. As emphasized by Nilsson (2014), phages intended to be used in phage therapy need to be well characterized, produced under appropriate conditions to obtain the required titer, validated, purified, and approved for clinical use. This will create a chance to minimize the problem of bacterial resistance to antibiotics. Thus, it is necessary to conduct phase III clinical trials (Vandenheuvel et al., 2015).

Table 1 summarizes phage topical application in the therapy of skin infections. All the described studies (**Table 1**) concern infections caused by bacteria other than *P. acnes*. These findings show that topical phage therapy in both animals and humans may be effective, so there are strong indications supporting the potential of *P. acnes* phages to also treat acne.

PHAGES ACTIVE AGAINST *PROPIONIBACTERIUM ACNES*

Bacteriophages are a viral component of the skin microbiome, but the knowledge about them in this environment is scant (Hannigan and Grice, 2013), probably because of the colonization of a specific ecological niche (anaerobic microenvironment). Since 1964 it has been known that bacteriophages are one of the components of the human skin community (Brzin, 1964). Webster and Cummins reported in 1978 that 18% of *P. acnes* isolates carried bacteriophages. In the skin microenvironment where there is a lack of bacteria other than *P. acnes* and their phages, *P. acnes* phages are proved to be closely related. The characteristics of described *P. acnes* bacteriophages are presented in **Table 2**.

Antibiotic resistance of *P. acnes* is emerging (Liu et al., 2014). Liu et al. (2014) isolated a bacterial strain from facial acne (nose skin) of a patient who had not previously applied an antibiotic for acne. It was observed that the isolated HL411PA1 strain was resistant to most antibiotics (including tetracycline, clindamycin, and erythromycin), which may confirm the presence of antibiotic resistance.

Liu et al. (2015) hypothesized that bacteriophages may play an important role in health and disease, because they are the main component of the human microbiota, and therefore they can modulate the bacterial community, especially in the case of human skin. In a pilosebaceous unit of skin it was found that the ratio between phages and bacteria is approximately 1:120, but it may vary (Fitz-Gibbon et al., 2013). Liu et al. (2015) analyzed 48 *P. acnes* phage metagenomes, and, based on this analysis, they found that human skin is colonized most commonly by one strain (Liu et al., 2015). It was also demonstrated that the transmission of phages between individuals is possible. Interestingly, phages active against *P. acnes* were isolated more frequently from skin of healthy volunteers than from patients suffering from acne, which may indicate that the role of phages in human skin is regulatory. One phage strain may dominate in the *P. acnes* phage population. It was also observed that some groups of tested phages were identified in different people, which may indicate that there is a pool of phages that may be shared in the human population. Furthermore, the transmission of skin phages is likely between closely related people. Based on their observations, the authors suggested that in designing phage therapy the composition of individual microbiome structure should be taken into consideration.

Marinelli et al. (2012) isolated 11 *P. acnes* phages from sebaceous follicles of healthy skin. The isolated phages were highly homogeneous and showed no genetic diversity, which the authors linked to their unique habitat. The tested phages had features (lytic lifecycle, lack of lysogeny-related genes, and the presence of endolysin-encoding genes) that make them an ideal tool for phage therapy of acne.

P. ACNES PHAGES' ABILITY TO KILL THE HOST STRAIN

The application of bacteriophages in the therapy of acne shows initial promise *in vitro* (Neely et al., 2008; Brown et al., 2016). *P. acnes* phages from skin of patients suffering from acne have been isolated by Brown et al. (2016). Phages active against *P. acnes* were found in both the gastrointestinal tract and the oral cavity (Willner et al., 2011; Sharon et al., 2013). The authors applied in a semi-solid preparation—Cetomacrogol cream, with concentration of 2.5×10^8 pfu/ml per gram—phages that remained active for 90 days when the preparation was stored in a light-protected place at 4°C (Brown et al., 2016). The non-ionic cream-based preparation was chosen because it excludes potential occurrence of interactions of ions with phage particles. These properties, especially stability and its easy-to-use form, ensure this preparation's potential for topical treatment of skin *P. acnes* infections. Benefits were observed from using the cream form, which can reduce the impact of harmful side-effects that are observed in common therapy of acne. Moreover,

TABLE 1 | Topical phage therapy of infection.

Pathogen	Model	Outcome	Result of the therapy
Staphylococcus aureus	Early studies of the application of phage therapy in dermatology in 143 patients with purulent skin infections.	Phage application as direct injection into the wound and surrounding tissue.	The best results were observed in patients with acute infections of deep skin. In the studied group of patients successful treatment was observed in 75%, improvement in 7.7%, and no effect of the therapy was observed only in 4.9% of the treated patients (Beridze, 1938).
Staphylococcus	55 patients with furunculosis.	Oral and local phage administration.	In all cases good therapeutic results were obtained (Ślopek et al., 1987).
Staphylococcus, *Pseudomonas*, *Klebsiella*, *Proteus*, *Escherichia*	Studies concerned 31 patients with suppurative skin infections.	The treatment lasted 2–16 weeks.	During the treatment an improvement with suppression of local inflammation, faster healing of ulcers, and eradication of bacteria was observed. Good therapeutic effects were obtained in the case of 25 patients (16 with outstanding results, 7 with marked improvement, and 2 with transient improvement; Cisło et al., 1987).
Klebsiella pneumoniae B5055	Mouse model of burn wound infection.	A single dose of topical application of the Kpn5 phage suspended in 3% hydrogel (at MOI of 200) used as ointment.	Mice treated with only a single dose of phage showed a significant reduction in animals' mortality (66%) compared to the control group (Kumari et al., 2010).
Staphylococcus aureus, Pseudomonas aeruginosa, *Acinetobacter baumannii*.	Animal models of diabetic cutaneous wound infection.	Topical administration in combination with wound debridement	A decrease in bacterial counts and improved wound healing in a rodent model of *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections. The therapy was not as effective against *Acinetobacter baumannii*. Bacteriophage treatment may be effective in resolving chronic infections also when applied in combination with wound debridement (Mendes et al., 2013).
Staphylococcus, *Streptococcus*, *P. aeruginosa*	Patients with wounds/ulcers.	Local administration of PhagoBioDerm, which contains ciprofloxacin, α-chymotrypsin benzocaine, and bacteriophage based on biodegradable poly(ester amide)s matrix.	Resulted in healing in the case of 70% of patients. It was associated with elimination of and/or reduction in pathogenic bacteria in the ulcers. This slow-release biopolymer was safe and of possible benefit in the management of refractory wounds, and the apparent utility of bacteriophages was supported in this setting (Markoishvili et al., 2002).
	Prospective, randomized, double-blind, controlled phase I study for the safety and efficacy of treatment of venous leg ulcers was conducted by the Southwest Regional Wound Care Centre in Lubbock, Texas, USA (in 2006–2008).	Once a week for 12 weeks topical application of a cocktail of 8 lytic bacteriophages against *P. aeruginosa*, *S. aureus*, and *E. coli*. (named WPP-201), developed by Intralytix Inc., USA.	No safety concerns regarding bacteriophage treatment (Rhoads et al., 2009).
P. aeruginosa	Twenty-four patients suffering from otitis media caused by antibiotic refractory *P. aeruginosa.* A double-blind, placebo-controlled initial phase I/II clinical trial targeting chronic external ear infections (in 2006–2007).	Application of bacteriophage mixture (Biocontrol Ltd., UK)	The results of the treatment of half of them treated with a single dose of bacteriophage mixture confirmed that phage administration was safe. A significant reduction of clinical symptoms at day 42 in the bacteriophage treated group was observed (55% of total clinical score at day zero) compared to the control group (104%). It was accompanied by a 76% decrease in mean count of bacteria in samples taken from the patients' ears 6 weeks after phage application, whereas in controls a 9% increase was observed (Wright et al., 2009).
E. coli or *P. aeruginosa*	*E. coli* or *P. aeruginosa* burn wound infections. A phase I/II randomized multi-center clinical trial involving 11 different burn units located in France, Belgium and Switzerland. This four-arm study involves 220 patients.	Two topically applied therapeutic phage cocktails (PP0121 and PP1131)	Its primary endpoint is the time for reduction of the targeted bacterial load in wound burns with a specifically designed microbiological procedure (Gabard et al., 2015).

TABLE 2 | Described phages that are active against *Propionibacterium acnes.*

Phage symbol, total number of isolated phage strains	Classification in family	Brief characteristics	Host range and specificity of action	Possible use in phage therapy
PA6	*Siphoviridae*	Lytic (lack of lysogeny genes). Phage isolated from skin scrub wash sample from patient. Phage produces clear plaques with turbid centers.	Able to lyse *P. acnes* strains, but not able to lyse other strains that are a part of the skin microbiome: *Propionibacterium granulosum*, *Propionibacterium avidum*, *Staphylococcus epidermidis*, Corynebacterium bovis (Farrar et al., 2007)	High specificity only against *P. acnes* and lytic life cycle may predispose to use of this phage in the therapy of acne.
PAC1-PAC10	Not done	Pseudolysogenic life cycle. Phage isolated from bacteria from skin swab sample from patient. It did not contain bacterial virulence factors.	Lysis of *P. acnes* strains, but not lysis of *P. acidipropionici*, *P. avidum*, *P. cyclohexanicum*, *P. jensenii*, *P. thoenii*, *P. freudenreichii. Narrow lytic spectrum.*	In Cetomacrogol cream aqueous concentration of phage for potential application in topical treatment of acne (Brown et al., 2016).
PAD2-PAD48, PAS2-PAS52	*Siphoviridae*	Presence of pseudolysogeny. Do not confer superinfection immunity.	Species specific. Only infect *P. acnes*, not other strains closely related to *Propionibacterium* (Lood et al., 2008).	Probably bad candidate for phage therapy.
P1.1, P9.1, P14.4, P100A, P100D, P100.1, P101.A, P104.A, P105	*Siphoviridae*	Probably the presence of pseudolysogeny (lack of lysogeny-related genes) phages. Isolated from healthy subjects and patients with acne. Lack of genetic diversity.	Broad range of clinical isolates, phage immunity if present is connected with the presence of chromosomally encoded elements.	Marinelli et al. (2012) suggested use of endolysin—peptidoglycan hydrolases that are bacteriophage-encoded antimicrobial peptides to treat bacterial infection.
48 phages, e.g., PHL111M01, PHL071N05, PHL060L00, PHL073M02	*Siphoviridae*	Pseudolysogenic and/or life cycle. 21 phages were isolated from patients with acne, 27 from healthy volunteers. Phages have limited diversity in genome.	*P. acnes* strains (including clades IA-1, IA-2, IB-1, and IB2) were susceptible to all 15 tested phages. But strains of clade IB-3, II, II were highly resistant to phages. Moreover, *P. granulosum* and *P. avidum* were resistant to all tested phages. Two strains of *P. humerusii* were susceptible to all tested phages, one was susceptible to 10 from 15 phages. Activity of *P. acnes* phages includes bacterial strains that are closely related to *Propionibacterium* species.	The authors suggested that the isolated phages may be used in modulation of *Propionibacterium* populations in human skin (Liu et al., 2015).
9 phages: from P-a-1 to P-a-9	Not done	Lytic phages, lysogenic ones were not detected. P-a1 to P-a7 were isolated from plaques on *P. acnes* lawn, but P-a-8 and p-a-9 came from sewage.	Both Gram-positive and Gram-negative strains from genera other than *Propionibacterium* were not lysed by these bacteriophages (Zierdt, 1974).	Bacteriophages were used to distinguish *C. ances* from *C. avidium* and *C. granulosum.* Lytic life cycle may predispose to use of this phage in the therapy of acne.
15 phages	Polyhedral heads with flexible unsheathed tails	Lysogenic, induced with mitomycin C from 17% *of P. acnes* strains	Isolated from *P. acnes* of healthy individuals. Strains of *P. acnes* belonging to serotype I were more susceptible to phage than those from serotype II (Webster and Cummins, 1978).	Probably bad candidate for phage therapy because of lysogenic cycle.
12 phages	Not done	Lysogenic phages. Phages isolated from skin swab sample from patient.	Phage of varying host range, but none which lyses all subtypes of *P. acnes.* (Neely et al., 2008).	Bad candidate for phage therapy because of lysogenic cycle.

the use of Cetomacrogol moisturizing cream may allow the contact of phage particles—which are pharmaceutically active components—with skin areas infected with *P. acnes*, which may improve the effectiveness of the suggested therapy. In this study it was found that the cocktail of phages did not result in higher inhibition of bacteria when compared to application of a single

phage, but due to the potential reduction of bacterial resistance, its use was recommended.

It was demonstrated that phages may be formulated in a cream (O'Flaherty et al., 2005), as well as in a water-oil nanoemulsion (Esteban et al., 2014). The latter formulation—bacteriophage containing a nanoemulsion—caused bacteria eradication *in vivo* 10 days after preparation. Markoishvili et al. (2002) reported application of PhagoBioDerm, which contains ciprofloxacin, α-chymotrypsin benzocaine, and bacteriophage based on a biodegradable poly(ester amide)s matrix in patients with wounds/ulcers. Phage application was also demonstrated in humans using an antiseptic gel and paraffin-oil-based lotion to destroy *Acinetobacter baumannii* (Chen et al., 2013).

The treatment of acne with phages in different types of formulations, e.g., creams or liquids, may be important in reducing the overuse of antibiotics, which is the primary means of acne treatment.

APPLICATION OF ENZYMES ENCODED IN PHAGE GENOME AND PHAGE MODIFICATION

Marinelli et al. (2012) found in phage genomes regions that encode phage endolysin, which is conserved in all tested *P. acnes* phages. These enzymes probably bind to essential elements of the *P. acnes* cell wall and may kill a broad range of *P. acnes* strains. They are essential proteins for the release of progeny, causing lysis of the host bacteria. The authors suggested that it may be possible to develop topical treatment of acne by the use of *P. acnes*-encoded endolysins. They are enzymes encoded in the bacteriophage genome with the ability to cause lysis of the bacterial cell wall peptidoglycan during the phage lytic cycle (Tišáková and Godány, 2014). The enzymes may create opportunities for the construction of genetically engineered enzymes for bacteria elimination, as well as experimental therapies. The detected endolysins in all the characterized *P. acnes* phages were found to be over 95% conserved at the amino acid level (Marinelli et al., 2012). This feature creates the possibility to use any endolysin successfully, destroying every *P. acnes* strain. Moreover, resistance to endolysins has not been observed so far (Nelson et al., 2012). The use of phage lysins as treatment against bacterial infections was demonstrated both *in vitro* and *in vivo*. The PlyG endolysin isolated from the γ phage was applied against *B. anthracis* by Schuch et al. (2002). Susceptibility of *B. anthracis* strains to the purified PlyG lysin indicated that this agent has a narrow bacteriolytic spectrum—especially with high activity against only *B. anthracis* strains. In bacterial culture, application of lysin caused morphological changes of bacterial cells and led to cell lysis. BALB/c mice were intraperitoneally infected with streptomycin-resistant *B. cereus* RSVF1 (with the same proven lytic activity of lysin as in the case of *B. anthracis* strains) and 15 min later were treated with 50 and 150 U of PlyG. The application of lysin significantly rescued mice in comparison to untreated animals. Bacterial resistance to PlyG was not observed *in vitro*, and the authors demonstrated that the RSVF1 strain that became resistant to the phage remained sensitive to PlyG.

Recently, we have suggested that phages armed with homing peptides should allow for their better tissue penetration and achieving *in situ* concentrations necessary for successful eradication of infection and control of inflammation (Górski et al., 2015). This methodology may also be relevant for phage treatment of infections associated with *P. acnes*.

Another approach suggests that phages may be used to improve antibiotic efficacy; in particular, phages may be a tool used for sensitizing antibiotic-resistant bacteria by introducing sensitivity genes (e.g., rpsL ayrA) to bacteria, which may restore drug efficacy (Wikoff et al., 2000; Salmond and Fineran, 2015). The authors believe that antibiotics conjugated with phages may enable the delivery of the drug to target cells and increase the concentration of the drug at the site of infection (Salmond and Fineran, 2015). The issue of a combined treatment with phages and antibiotics has recently been addressed in some detail by Torres-Barceló and Hochberg (2016). This approach has been recommended by some authors (e.g., Chanishvili, 2016). However, we need more data to establish the real value of such therapy, and one should also note that the emergence of double-resistant phage mutants poses a potential threat (Torres-Barceló and Hochberg, 2016).

LIMITATIONS OF PHAGE USE FOR ELIMINATION OF *P. ACNES*

Bacteria have an adaptive immune system which is based on a region of DNA called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (*cas*) genes separated by short sequences (Rath et al., 2015; Maxwell, 2016). This system protects bacteria against viruses and other mobile genetic elements. It may be responsible for phage resistance in bacteria. Also *P. acnes* proved to have CRISPR elements, and its role in the phage resistance results from the correlation between these elements and spacer matches with the phage genomes, as suggested by Marinelli et al. (2012). This system, which has also been found in *P. acnes*, may cause phage ineffectiveness (Brüggemann and Lood, 2013).

Another limitation associated with the application of *P. acnes* phages is the high homogeneity within *P. acnes* phages, which may be beneficial with the use of phage formulations of broad activity and application of available ready to use phages of limited activity, avoiding phage typing. However, acquisition of resistance to one phage may result in resistance to others (Brüggemann and Lood, 2013). The effect of *P. acnes* phages' homogeneity may limit the efficacy of the phage therapy.

The term "pseudolysogeny" for *P. acnes* phages was first mentioned in 2011 by Lood and Collin (2011). Probably most, if not all, *P. acnes* phages characterized to date display pseudolysogeny (Farrar et al., 2007; Lood et al., 2008; Lood, 2011; Marinelli et al., 2012) and form turbid plaques. Pseudolysogeny is an unstable state in which the phage genome is not integrated into the bacterial genome (Lood and Collin, 2011). Without cell lysis, the phage genome exists in the host cell as an episome,

with superinfection immunity of bacteria from phage infection, and phage DNA exists as a subpopulation. Both the lysogenic and pseudolysogenic cycle do not cause direct lysis of bacteria, or may lead to phage resistance, and these phenomena are not beneficial from the therapeutic point of view. Phages specifically active against *P. acnes* strains may shape the skin microbiome composition and influence the health-disease balance. Moreover, they do not integrate into the host chromosome and transfer bacterial pathogenicity genes, which is an advantage in their use as therapeutics.

There are no data in the available literature regarding phage application against skin infections caused by *P. acnes* in an animal model, while the results obtained *in vitro* do not necessarily translate into the situation *in vivo*. This situation does not facilitate prompt clinical application of phages. On the other hand, phages intended for topical use in the treatment of acne could be used as a medical application which could make the process of their registration more straightforward.

The potential of phage therapy in the treatment of acne has been highlighted by a recent article that emphasized the need for more prudent use of antibiotics in this condition, as well as an urgent need to search for alternative treatments. The authors also draw attention to the changing concept of acne, where inflammation appears to play a prominent role in its pathology. This constitutes another strong argument for the potential application of phages in acne, as the anti-inflammatory action of phage therapy is well documented (Górski et al., 2012).

CONCLUDING REMARKS

Phage therapy offers a real chance for patients suffering from chronic infections caused by antibiotic-resistant bacteria. This therapy may replace or supplement conventional antibiotic therapy, help eliminate antibiotic resistance of bacterial strains, and eliminate deleterious effects of chemical antibiotics.

There is a need to develop phage therapy of acne, but more research is needed to understand bacteria-bacteriophage interactions in the skin community to obtain comprehensive knowledge on how to use phages to combat *P. acnes* when it becomes pathogenic (in the pathogenesis of acne vulgaris). Phages should gain acceptance and be widespread as an antibiotic supplement or an alternative in the treatment of infections caused by antibiotic-resistant bacteria, including those whose etiological agent is *P. acnes*. There are also some limitations of the potential application of anti-*P. acnes* phages. Those include their high homogeneity which may cause difficulties in identifying other phages if phage resistance develops. In addition, the lack of data on phage application in skin infections caused by *P. acnes* may delay such clinical application.

AUTHOR CONTRIBUTIONS

EJ and BW drafted the main part of the manuscript. MŻ prepared a part of the manuscript and **Table 2**. RM, SL, and MŁS prepared parts of the manuscript. AG gave support and conceptual advice at all stages of manuscript preparation. All authors revised the manuscript.

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Characterization of Novel Bacteriophages for Biocontrol of Bacterial Blight in Leek Caused by *Pseudomonas syringae* pv. *porri*

Sofie Rombouts[1,2], Anneleen Volckaert[3], Sofie Venneman[4], Bart Declercq[5], Dieter Vandenheuvel[6], Camille N. Allonsius[6], Cinzia Van Malderghem[2], Ho B. Jang[1], Yves Briers[1,7], Jean P. Noben[8], Jochen Klumpp[9], Johan Van Vaerenbergh[2], Martine Maes[2,10] and Rob Lavigne[1]*

[1] Laboratory of Gene Technology, Department of Biosystems, KU Leuven, Leuven, Belgium, [2] Unit Plant— Crop Protection, Institute for Agricultural and Fisheries Research, Merelbeke, Belgium, [3] Vegetable Research Centre (PCG), Kruishoutem, Belgium, [4] Research Station for Vegetable Production, Sint-Katelijne-Waver, Belgium, [5] Inagro, Rumbeke, Belgium, [6] Laboratory of Applied Microbiology and Biotechnology, Department of Bioscience Engineering, University of Antwerp, Antwerpen, Belgium, [7] Laboratory of Applied Biotechnology, Department of Applied Biosciences, Ghent University, Ghent, Belgium, [8] School of Life Sciences, Biomedical Research Institute and Transnational University Limburg, Hasselt University, Diepenbeek, Belgium, [9] Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland, [10] Lab. of Microbiology, Department of Biochemistry and Microbiology, Ghent University, Gent, Belgium

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University of the Sunshine Coast, Australia
Ian F. Connerton,
University of Nottingham, UK

***Correspondence:**
Rob Lavigne
rob.lavigne@kuleuven.be

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Pseudomonas syringae pv. *porri*, the causative agent of bacterial blight in leek (*Allium porrum*), is increasingly frequent causing problems in leek cultivation. Because of the current lack of control measures, novel bacteriophages were isolated to control this pathogen using phage therapy. Five novel phages were isolated from infected fields in Flanders (vB_PsyM_KIL1, vB_PsyM_KIL2, vB_PsyM_KIL3, vB_PsyM_KIL4, and vB_PsyM_KIL5), and were complemented with one selected host range mutant phage (vB_PsyM_KIL3b). Genome analysis of the phages revealed genome sizes between 90 and 94 kb and an average GC-content of 44.8%. Phylogenomic networking classified them into a novel clade, named the "KIL-like viruses," related to the *Felixounalikevirus* genus, together with phage phiPsa374 from *P. syringae* pv. *actinidiae*. *In vitro* characterization demonstrated the stability and lytic potential of these phages. Host range analysis confirmed heterogeneity within *P. syringae* pv. *porri*, leading to the development of a phage cocktail with a range that covers the entire set of 41 strains tested. Specific bio-assays demonstrated the *in planta* efficacy of phages vB_PsyM_KIL1, vB_PsyM_KIL2, vB_PsyM_KIL3, and vB_PsyM_KIL3b. In addition, two parallel field trial experiments on three locations using a phage cocktail of the six phages showed variable results. In one trial, symptom development was attenuated. These data suggest some potential for phage therapy in controlling bacterial blight of leek, pending optimization of formulation and application methods.

Keywords: *Pseudomonas syringae* pv. *porri*, leek bacterial blight, phage therapy, KIL-like viruses, phylogenomics

INTRODUCTION

In recent years, an increase in the prevalence of leek bacterial blight was noted in Flanders, Belgium. The disease is caused by the bacterial pathogen *Pseudomonas syringae* pv. *porri*, which was first reported in the United Kingdom by Lelliott (1952). Two decades later, the species was isolated in New Zealand by Hale (1975) who further described the pathogen. Later, the bacteria was classified as a new pathovar, *P. syringae* pv. *porri*, based on extensive research by Samson et al. in France in 1998. Currently, the disease has spread globally and is reported in The Netherlands (Janse, 1982; van Overbeek et al., 2010), Italy (Varvaro, 1983), the United States (Koike et al., 1999), Australia (Noble et al., 2006), Greece (Glynos and Alivizatos, 2006), Japan (Goto, 1972), and Korea (Myung et al., 2011, 2012). Typical symptoms include leaf curling and yellowing of the middle vein in young plants and water soaked spots on older leaves and flowering stems (Samson et al., 1998; Noble et al., 2006). Leek (*Allium porrum*) is the major host, but the pathogen has also been diagnosed on onion (*Allium cepa*) and shallot (*Allium cepa* var. *aggregatum*; Noble et al., 2006). The disease is known to be transmitted by seed (Koike et al., 1999; Ikene et al., 2003), but crop waste also plays a role in contaminating new leek plants in the field (van Overbeek et al., 2010).

The recent rise in leek bacterial blight is consistent with a growing number of Flemish farmers obtaining leek transplants from plant nurseries. High plant densities in those nurseries, combined with plant manipulations such as irrigation and mowing, promote dissemination of the pathogen among the transplants (Koike et al., 1999). In a previous study, the causative agent of recent leek blight epidemics in Flanders was examined, leading to the proposed subdivision of the strains into two groups, with small genomic differences (Rombouts et al., 2015). Knowledge on this intra-pathovar diversity is important to determine the specificity of phages isolated and characterized within this study.

To date, disease management in leek production mainly consists of prevention. Clean seeds, a strict hygiene in plant nurseries and the use of more tolerant varieties are important control measures. However, once the first symptoms of bacterial blight appear, no solution is available because of the ban on streptomycin derivatives to control bacterial plant diseases in the European Union. The use of copper-based agrichemicals is also undesirable in view of ecotoxicity and bacterial resistance development (Cooksey, 1994; Pietrzak and McPhail, 2004; Hwang et al., 2005). Therefore, the use of phage therapy constitutes an attractive alternative. Bacteriophages are viruses that specifically infect bacteria, their replication resulting in the lysis of their bacterial host and the release of newly formed viral particles. Several reviews have previously been published, highlighting the possibilities and limitations of phage therapy in plant disease control (Gill and Abedon, 2003; Jones et al., 2007; Balogh et al., 2010). In agriculture, phage therapy research has been conducted for pathogens in other crops including "*Dickeya solani*" (Adriaenssens et al., 2012), *Erwinia amylovora* (Gill et al., 2003; Boulé et al., 2011), *Pectobacterium carotovorum* (Ravensdale et al., 2007), *Ralstonia solanacearum* (Fujiwara et al., 2011), *Xanthomonas axonopodis* pvs. *citri* and *citrumelo* (Balogh et al., 2008), and *P. syringae* pv. *actinidiae* (Frampton et al., 2014). To date, phage research resulted in a limited number of commercial phage-based products for agricultural use. These products are for control of two tomato pathogens, *Xanthomonas campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato* (AgriPhage; Flaherty et al., 2000; Balogh et al., 2003) and against potato rot (Biolyse, APS Biocontrol Ltd.).

Phages infecting *P. syringae* pv. *porri* have not yet been described. In this manuscript, we focus on the isolation of novel phages which cover the bacterial diversity present in Flemish leek production. Extensive *in vitro* characterization of the phages was followed by bio-assays and field trials to evaluate the potential of these phages for the biocontrol of bacterial blight in leek.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in **Table 1**. *P. syringae* pv. *porri* strains were isolated from fields in Flanders and covered the existing diversity. Their identification and characterization was previously published (Rombouts et al., 2015). These isolates were supplemented with P55, an isolate from soil from the study of van Overbeek et al. (2010), and reference strains of *P. syringae* pv. *porri* from the Collection Française de Bactéries Phytopathogènes (CFBP; Beaucouzé, France), including the type strain and a set of phylogenetic related strains. Bacteria were grown in lysogeny broth with reduced salt concentration (LB_{rs}) (0.5 g/l NaCl) at 26°C, or on LB_{rs} plates supplemented with 1.5% agar. LB_{rs} with 0.7% agar (soft agar) was used for the plate overlays.

Bacteriophage Isolation, Amplification, and Purification

Phages were isolated from soil samples taken in 2011 and 2012, from the same fields from which the *P. syringae* pv. *porri* strains were isolated. To enrich for phages, a bacterial host (LMG 28495 or LMG 28596) was grown in 25 ml LB_{rs} at 26°C up to exponential growth phase and 5 g of soil sample was added. After overnight incubation, 250 µl chloroform was added and incubation was continued for 1 h to lyse all bacterial cells. Next, the mixture was centrifuged (30 min, 4500 rpm) in a Sorvall Legend RT+ centrifuge with swing-out 4-place rotor, type 75006445 (Thermo Scientific, Waltham, MA, USA). The supernatant was filtered through a Millex® syringe filter with a 0.45 µm pore size (Merck Millipore Ltd.) and 10 µl was spotted on a soft agar layer that contained the bacterial host. Lysis zones were picked up with sterile toothpicks and suspended in phage buffer (10 mM Tris-HCl; pH 7.5; 10 mM $MgSO_4$; 150 mM NaCl). These suspensions were plated by pooling 250 µl overnight bacterial host culture, 100 µl phage suspension and 4 ml LB_{rs} overlay agar, according to the overlay agar technique (Adams, 1959). After overnight incubation at 26°C, single plaques were picked up again. Three successive single plaque isolations were performed to achieve pure phage isolates. Based on their DNA restriction pattern (data not shown) different phages were selected for further analysis.

One bacteriophage (KIL3b) was obtained after a co-cultivation experiment. This experiment consisted of the infection of an

TABLE 1 | *P. syringae* pv. *porri* strains and related *P. syringae* pathovars with their phage sensitivity and BOX-PCR profiles.

Bacterial strain[a]	Plant origin	Origin[b]	Year[c]	Identification	KIL 1	KIL 2	KIL 3	KIL 4	KIL 5	KIL 3b	BOX-PCR
•GBBC 1427	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	–	–	–	+	+	–	
•GBBC 1428	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	–	–	–	+	+	–	
•GBBC 1438	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	–	–	–	+	+	–	
•GBBC 1444	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	–	–	–	+	+	–	
•LMG 28496	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	–	–	–	+	+	–	
×GBBC 715	*Allium porrum*	BE	2001	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 722	*Allium porrum*	BE	2001	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 728	*Allium porrum*	BE	2002	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 747	*Allium porrum*	BE	2002	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1088	*Allium porrum*	MA	2011	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1089	*Allium porrum*	MA	2011	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1090	*Allium porrum*	MA	2011	*P.s.* pv. *porri*	–	–	–	–	–	+	
×GBBC 1113	*Allium porrum*	BE	2003	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1165	*Allium porrum*	BE	2004	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1166	*Allium porrum*	BE	2004	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1170	*Allium porrum*	BE	2004	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1184	*Allium porrum*	BE	2004	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1255	*Allium porrum*	BE	2005	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1256	*Allium porrum*	BE	2005	*P.s.* pv. *porri*	–	–	–	–	–	+	
×GBBC 1267	*Allium porrum*	BE	2005	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1269	*Allium porrum*	BE	2005	*P.s.* pv. *porri*	–	–	–	–	+	+	
×GBBC 1272	*Allium porrum*	BE	2005	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1273	*Allium porrum*	BE	2005	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1277	*Allium porrum*	BE	2006	*P.s.* pv. *porri*	+	–	–	–	–	+	
×GBBC 1286	*Allium porrum*	BE	2006	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1311	*Allium porrum*	BE	2007	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1424	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1426	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1433	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1434	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1435	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1452	*Allium porrum*	BE	2012	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1459	*Allium porrum*	BE	2013	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1462	*Allium porrum*	BE	2013	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1893	*Allium porrum*	NL	2013	*P.s.* pv. *porri*	+	+	+	+	+	+	
×GBBC 1894	*Allium porrum*	NL	2013	*P.s.* pv. *porri*	+	+	+	+	+	+	
×LMG 28495	*Allium porrum*	BE	2011	*P.s.* pv. *porri*	+	+	+	+	+	+	
×P55[d]	soil of leek field	NL	2010	*P.s.* pv. *porri*	+	–	–	–	–	+	
×CFBP 1908[PT]	*Allium porrum*	FR	1978	*P.s.* pv. *porri*	+	+	+	+	+	+	
×CFBP 1687	*Allium porrum*	GB	1949	*P.s.* pv. *porri*	+	+	+	+	+	+	
×CFBP 1770	*Allium porrum*	NZ	1973	*P.s.* pv. *porri*	+	+	+	+	+	+	
CFBP 3228[PT]	*Oryza sativa*	JP	1983	*P.s.* pv. *oryzae*	–	–	–	–	–	–	
CFBP 1634[PT]	*Coffea arabica*	BR	1958	*P.s.* pv. *garcae*	–	–	+	–	–	+	
CFBP 1674[PT]	*Avena sativa*	–	1958	*P.s.* pv. *striafaciens*	–	–	–	–	–	–	
CFBP 2216[T]	*Avena sativa*	GB	1958	*P.s.* pv. *coronafaciens*	–	–	–	–	–	–	
CFBP 4117[PT]	*Zizania aquatica*	US	1983	*P.s.* pv. *zizaniae*	–	–	–	–	–	–	

The two symbols (• and ×) indicate two BOX-PCR pattern groups. The arrow gives the position of the discriminative band. Light and dark green: lysis after 24 and 48 h, respectively and yellow: lysis from without. [a]*GBBC: culture collection of plant pathogenic bacteria at ILVO; CFBP, Collection Française de Bactéries Phytopathogènes; INRA Angers; LMG, Belgian Coordinated Collections of Microorganisms at the Laboratory of Microbiology of Ghent University with* [T] *as type strains and* [PT] *as pathovar reference strains.* [b]*Geographical origin.* [c]*Year of isolation.* [d]*received from van Overbeek et al. (2010).*

TABLE 2 | Bacterial host of the new bacteriophages and phage genome characteristics determined by bioinformatic analysis.

Phage name	GenBank accession number	Bacterial host	Genome length (bp)	GC content (%)	# ORFs	# tRNAs	Terminators	Bacterial promoters
KIL1	KU130126	CFBP 1687	90695	44.86	159	5	18	57
KIL2	KU130127	CFBP 1687	92466	44.79	163	9	17	59
KIL3	KU130128	CFBP 1687	92089	44.74	161	5	17	61
KIL4	KU130129	LMG 28496	92825	44.89	167	9	18	60
KIL5	KU130130	LMG 28496	93384	44.97	169	9	18	59
KIL3b	KU130131	CFBP 1687	92099	44.72	161	5	17	61

exponentially growing liquid culture of *P. syringae* pv. *porri* strain CFBP 1687 with phage KIL3 at a multiplicity of infection (MOI) of 0.01. For 4 weeks, 40 µl of the liquid culture was transferred to a tube with 4 ml fresh LB_{rs} medium every 2 to 3 days and incubated at room temperature. Supernatant of every two-day-old liquid culture was spot tested against the bacterial strain P55 to select for a broadened lytic activity as none of the other phages were able to lyse this strain.

Phages were amplified by plating 10^5 plaque forming units (pfu) per plate together with their bacterial host on a soft agar overlay plate. After overnight incubation at 26°C, the soft agar was scraped off and suspended in phage buffer. Soft agar and cell debris were subsequently removed by centrifugation (20 min, 4000 rpm) in a Sorvall Legend RT+ centrifuge. The supernatant was filtered over a 0.22 µm membrane Millex® syringe filter (Merck Millipore Ltd.), after which polyethylene glycol (PEG) 8000 (Sigma-Aldrich) containing 1 M NaCl was added to the suspension to a final concentration of $8\%_{w/v}$. After minimum 3 h incubation at 4°C, phages were precipitated by centrifugation (30 min, 4600 rpm) and the pellet was dissolved in 1 ml phage buffer.

Phage purification was performed using CsCl-gradient ultracentrifugation. A phage suspension (15 ml) with a minimum concentration of 10^{10} pfu/ml was layered on a CsCl step gradient (1.33, 1.45, 1.50, and 1.70 CsCl g/cm^3) and centrifuged (3 h, 140.000 × g) with a Beckman Coulter Optima L-90K ultracentrifuge (rotor type SW28) (Brea, CA, USA). All phages were collected at the interface between the 1.45 and 1.50 g/cm^3 density layer and dialyzed three times for 30 min against 300 volumes of phage buffer.

Electron Microscopy

To obtain transmission electron microscopy (TEM) images of the bacteriophages, dilutions of the samples were spotted on carbon coated grids (Quantifoil, Großlöbichau, Germany) after glow-discharge, and negatively stained with 2% uranyl acetate. A Philips CM12 microscope was used at 120 kV acceleration voltage. Images were produced using a Gatan Orius 1 k camera.

Host Range Analysis and General Characterization

Phage host range was tested by spotting a phage suspension on plates with a soft agar layer supplied with a specific bacterial isolate. For all the phages, three different concentrations, ranging from 10^2 to 10^6 pfu/ml, were tested to determine infectivity. The bacterial strains used in the host range assay are listed in **Table 1**.

Adsorption experiments were performed according to Adriaenssens et al. (2012). Briefly, bacteria and phage were mixed at an MOI of 0.0001. Next, samples were taken every minute and bacteria were immediately lysed by adding chloroform. The upper phase was titred to determine the number of non-adsorbed phages.

Killing curves at different MOIs were generated by infecting a bacterial culture at an optical density at 600 nm (OD_{600}) of 0.3. The OD_{600} of infected and uninfected cultures was monitored every 20 min during 6 h. OD_{600} results were the average of three independent biological repeats.

Phage stability was tested by incubating a phage suspension of 10^6 pfu/ml in phage buffer at different temperatures (−20, 4, 16, 30, 37, and 50°C) and at different pH ranging from 1 to 13 (universal buffer consisting of 150 mM KCl, Janssen Chimica, Geel, Belgium; 10 mM KH_2PO_4, VWR International, Leuven, Belgium; 10 mM sodium citrate, Acros Organics; 10 mM H_3BO_3, Acros Organics; adjusted to pH 1–13 with NaOH, or HCl). Infectivity of the phages at different temperatures was tested by incubating soft agar plates with spots of different phage concentrations at room temperature (±21°C), 26 and 30°C and counting the infective phage titers.

The frequency of bacterial resistance was determined using an adaptation of a previously developed method (Beale, 1948). Using the agar overlay method, phage and bacteria were plated at MOI 1 and MOI 10, in order to lyse all bacteria on the plate. After 72 h of incubation, emerging colonies were counted and about 20 colonies were cultured and re-tested for resistance against the phages in **Table 2**. Phage-resistant isolates were plated on Pseudomonas agar F (PAF, Becton Dickinson) supplemented with glycerol to check for fluorescence under UV-light and analyzed with a multiplex PCR containing specific primers (Ineke Wijkamp, Rijkzwaan, personal communication) to confirm their *P. syringae* pv. *porri* identity.

Lysogeny in these resistant bacteria was tested as previously described (Petty et al., 2006). Supernatant from overnight cultures of resistant bacteria were collected and tested for the spontaneous release of phage particles by spotting 5 µl drops onto soft agar containing the different bacterial hosts. The supernatants from CFBP 1687 and LMG 28496 were used as negative controls, and pure phage solutions (10^6 pfu/ml) were used as positive controls for plaque formation.

Genome and Proteome Analysis

DNA Isolation and Sequencing

Contaminating bacterial DNA was removed from phage lysates by DNase treatment. Upon inactivation of the DNase by the addition of EDTA, phage particles were disrupted by adding SDS and proteinase K. The released phage DNA was purified by standard phenol-chloroform extraction and subsequent ethanol precipitation according to Sambrook and Russell (2001). DNA concentration and purity was verified with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). DNA integrity and genome size was evaluated on an 0.8% agarose gel. High throughput sequencing was done using the Illumina MiSeq platform. A 2*250 bp paired-end library was prepared for each sample, tagged with a unique adapter sequence. The quality of each library preparation was controlled using the Agilent Bioanalyzer. All library preps were equally pooled and sequenced. After processing, the reads of every library prep were assembled in a single contig with a general coverage over 1000x using the CLC Bio Genomics Workbench de novo assembly algorithm (version 7.5.1) (Aarhus, Denmark).

"*In silico*" Analysis

The genomes were scanned for open reading frames (ORFs) with GeneMark.hmm and GeneMarkS software (Lukashin and Borodovsky, 1998; Besemer et al., 2001). Shine-Dalgarno sequences were verified manually upstream of each annotated ORF. Functional annotation was carried out by comparing translated ORFs in a Blastp analysis (Altschul et al., 2005) against the nonredundant GenBank protein database and with the Protein Homology/analogY Recognition Engine v2.0 (PHYRE2) (Kelley and Sternberg, 2009). tRNAs were detected with the programs ARAGORN and tRNAscanSE (Lowe and Eddy, 1997; Laslett and Canback, 2004). Rho-independent terminators were predicted with ARNold, a search program that combines two complementary programs: RNAMotif and Erpin (Gautheret and Lambert, 2001; Macke et al., 2001). Probable promoter sequences were identified by looking for conserved intergenic motifs in the 100 bp upstream of every ORF with MEME and PHIRE (Lavigne et al., 2004; Bailey et al., 2009).

Protein Clustering and Phylogenetic Analysis

Homologous proteins, deduced from their DNA sequences, were examined in the phages KIL1, KIL2, KIL3, KIL4, KIL5, KIL3b, and a number of fully sequenced bacteriophages using HMMER (version3.0; http://hmmer.janelia.org), together with the ACLAME search (version0.4; http://aclame.ulb.ac.be; as of June 2015). In this manner, we retrieved a total of 4632 predicted protein sequences represented in the genomes of the 26 relevant phages (Table S2). We performed pairwise similarity comparisons for each predicted protein by using the ACLAME database with the database of "viruses" and an *E*-value < 0.001 (Lima-Mendez et al., 2008). The remaining proteins which could not be assigned into any ACLAME protein family were defined as the unclassified protein families (UPFs) as previously described (Jang et al., 2013). The protein families that were common to all 32 representative phage genomes were identified and their phylogenetic relationships were inferred. After multiple sequence alignment of the conserved proteins using CLUSTALW2, non-informative positions were excluded with the BMGE software package (Criscuolo and Gribaldo, 2010). The two alignments were then concatenated into a FASTA file and a phylogenetic tree was built with MEGA5 (version 5.2.1; http://www.megasoftware.net/; Tamura et al., 2011) using a Jones–Taylor–Thornton (JTT) model. To estimate the robustness of the trees, we used the maximum-likelihood algorithm provided with bootstrap support ($n = 1000$ replicates).

Proteome Analysis

Structural proteins of phages KIL3 and KIL5 were identified by SDS-PAGE gel electrophoresis, isolation of gel bands, and subsequent trypsinization and ESI-MS/MS as described previously (Lavigne et al., 2009a). The resulting data were analyzed using Mascot (version 2.3.01) and Sequest (version 1.2.0.208), against a database containing all possible ORFs based on the DNA sequence of the phages.

Phage Therapy Experiments

Bio-Assay

In planta activity of the phages was tested by injecting phage and bacterial suspensions into leek leaves. Leek plants of the disease susceptible cultivar Krypton (Nunhems) were grown in separate pots until full development of three leaves. Next, 0.1 ml of bacterial suspension was injected with a syringe into the middle of the leaf. Subsequently, 3 cm above the bacterial injection place 0.1 ml of phage suspension (10^9 pfu/ml) was injected (Supplementary Figure 1). In the first bio-assay all 41 *P. syringae* pv. *porri* strains (10^7 CFU/ml) were inoculated in leek leaves (three inoculated leaves/strain) and all phage types (10^9 pfu/ml) were co-inoculated with a *P. syringae* pv. *porri* representative (eight inoculations/phage-bacteria combination). This experiment was repeated using the disease susceptible cultivar Striker (Bejo) and a lower bacterial concentration (10^6 CFU/ml). Phages KIL1, KIL2, KIL3, and KIL3b (10^9 pfu/ml) were co-inoculated with bacterial strain CFBP 1687 and phages KIL4 and KIL5 with LMG 28496 (10 inoculations/phage-bacteria combination). Plants were covered for 48 h with a plastic bag to maintain humid conditions and kept at a temperature of 25°C. Lesion lengths were measured after 10 days. Injections with only bacteria, phage or buffer served as controls.

Field Trials

In a first trial, leek transplants were treated with phages before they were planted in an infested field. First, the field was infested by spraying a solution of CFBP 1687 with a concentration of 10^6 CFU/ml on the soil at a rate of 1000 l/ha, at 1.5 bar and with 0.01% Tween® 20 as surfactant. The next day, before planting, leek plants were submerged in a solution containing a mixture of the six different phages, each at a concentration of 10^7 pfu/ml. Control plants were planted without treatment. Four blocks of the phage-treated and non-treated plants were randomly distributed over the field, with each block containing 300 plants. At multiple time points after planting, disease incidence (number of damaged plants) as well as disease severity (% of leaf surface affected) was measured for 20–50 plants per block. This trial

was performed simultaneously on three different locations in Flanders: Kruishoutem, PCG (N 50.94337°, E 3.52710°), Sint-Katelijne Waver, PSKW (N 51.078120°, E 4.528180°), and Beitem, Inagro (N 50.901508°, E 3.124464°).

In a second trial, leek transplants were planted in a non-infested field. Two months after planting, bacterial infection was performed with strain CFBP 1687. A suspension with a concentration of 10^6 CFU/ml was spread over the plants at a rate of 1000 l/ha, at 1.5 bar and with 0.01% Tween® 20 as surfactant. The next day, a mix of the six different phages, each at a concentration of 10^9 pfu/ml and supplemented with 0.01% Tween® 20, was sprayed over the plants, in a way that each plant was covered with on average 10^8 pfu of each phage after spraying. Plants were covered with plastic for 48 h and assessed as described. This trial was also performed in parallel on the three different locations in Flanders mentioned above.

Statistical Analysis of Data

Statistical analyses were performed with IBM SPSS Statistics 20. For the bio-assays, normality of the data was assessed with the Kolmogorov-Smirnov and Shapiro-Wilk test at a significance level of 0.05. Homogeneity of variances was tested using the Levene's test. When the data were distributed normally and homoscedasticity was proven, an one-way ANOVA test was performed to check for differences. If the tests showed significant differences, the data were further analyzed with a *post-hoc* Tukey HSD test. To test for differences between groups of not normally-distributed data, a Kruskal-Wallis non-parametric test was used. When differences were present, Mann-Whitney U non-parametric test was used to compare the groups, paired two by two. For the field trials, the statistical program ARM (version 2015.3) was used. This program, which is designed specifically for field trials, uses the Bartlett's test to test the homogeneity of variances and the Kurtosis and Skewness tests to check normality. One-way ANOVA with the *post-hoc* Duncan test was used for normal distributed and homoscedastic data. As a non-parametric test, the Friedman test was applied to check for differences between several variables.

RESULTS

Phage Isolation and TEM

Phages were isolated from soil samples taken in 2011 and 2012 from the same fields from which *P. syringae* pv. *porri* isolates were obtained (**Table 2**). After enrichment of the phages present in the soil, their capacity to lyse *P. syringae* pv. *porri* strain CFBP 1687 was tested. Based on their differential DNA restriction pattern (data not shown) phages were selected for further analysis. By this method, five novel phages were discovered for *P. syringae* pv. *porri* and the phages were named KIL1, KIL2, KIL3, KIL4, and KIL5 (referring to the involved research institutes KU Leuven and ILVO) with scientific names vB_PsyM_KIL1 etc. as proposed by Kropinski et al. (2009). The host strains used for amplification and characterization of each of these phages are indicated in **Table 2**.

TEM images of a phage representative revealed that they belong to the *Myoviridae* family with morphological similarity to the Pseudomonas phage *PB1* (genus *Pbunalikevirus*), as shown for phage KIL1 in **Figure 1**. The other analyzed phages have an identical morphology.

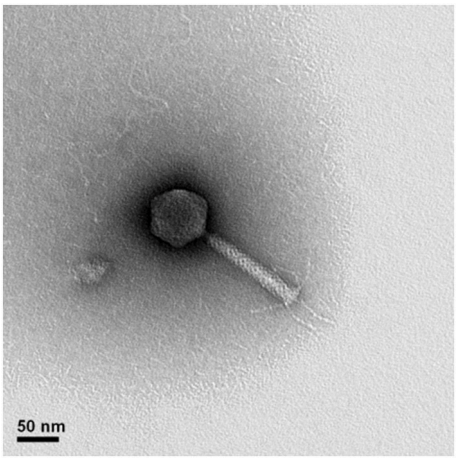

FIGURE 1 | EM picture of phage KIL1.

Host Range Analysis

To investigate the specificity of the phages, a host range analysis was performed (**Table 1**), by using standard serial dilution spotting assays to distinguish infection from lysis from without phenomena, as described previously (Adriaenssens et al., 2012). Some of the bacterial isolates could not be infected by our five phage isolates from soil, therefore the host-range mutant phage KIL3b was developed. The results indicate that the bacterial strains can be divided in three groups. One group consists of five field isolates from the year 2012 and was only infected by phages KIL4 and KIL5. A second group of strains was infected by all the investigated phages and a third group of strains only showed lysis after 48 h and by a limited number of phages. None of the pathovars related to *P. syringae* pv. *porri* was infected by the phages demonstrating their specificity for pathovar *porri*. However, *P. syringae* pv. *garcae* (CFBP 1634) showed "lysis from without" at phage concentrations of 10^6 pfu/ml. In addition, none of the phages infected all *P. syringae* pv. *porri* strains tested but the combined host range of the phages covers all 41 isolates tested. A previous BOX-PCR analysis on DNA of all the bacterial strains used in the host range analysis provided information about their genomic diversity and identified two groups within *P. syringae* pv. *porri* (Rombouts et al., 2015). These two groups were confirmed by phage profiling, suggesting that differences in the bacterial genome are responsible for the observed host range. Two phages, KIL3 and KIL5 were selected for further characterization.

General Characteristics

To assess the infection parameters, adsorption experiments were performed for phage KIL3. On average, 84% of phages were irreversibly adsorbed to the host cell after 1 min and 99% after 6 min with an adsorption constant k [$k = (2.3/(B^*t))^*\log(P_0/P)$, with B the bacterial titer at time zero, P the phage titer and t

the time] at 1 min of 7.52×10^{-9} ml/min. Compared to other *Myoviridae* phages, this is slower than reported for phage T4 (2.4×10^{-9} ml/min) but comparable to the adsorption constants of phages LIMEstone1 (9.53×10^{-9} ml/min) and LIMEstone2 (2.05×10^{-8} ml/min) infecting *Dickeya* (Kasman et al., 2002; Adriaenssens et al., 2012).

Killing curves generated by infecting an exponentially growing cultures of *P. syringae* pv. *porri* CFBP 1687 and LMG 28496 with phages KIL3 and KIL5, respectively, at different MOI demonstrated their virulence (Supplementary Figure 2). Optical density of the bacterial cultures at 600 nm decreased after 80 and 100 min for phage KIL3 and KIL5, respectively. KIL5 showed a later but steeper decline reaching $OD_{600} < 0.1$ after 180 min instead of 220 min for KIL3. No rise in OD_{600} appeared within the 5 h of monitoring indicating that resistance did not develop at that point.

The influence of temperature and pH on the viability was tested. As shown for KIL3 and KIL5 (representatives of the two phage clades in the cocktail), the phages were stable between 4 and 37°C in phage buffer for 24 h, but after 24 h of incubation at 50°C, a two and one $\log_{10}$ unit decrease was noted for phage KIL3 and KIL5, respectively. After freezing, all viable KIL3 phages were lost, while the titer of KIL5 decreased by three log10 units. Phages were stable from pH 4 to 12 for 24 h.

An analysis of the optimal infection temperature revealed that none of the phages are able to infect their host when grown at temperatures of 30°C. Phages KIL1, KIL2, KIL3, KIL4, and KIL5 were able to infect at temperatures of 26°C and room temperature (±21°C), and KIL3b only infected at room temperature.

For two phage-host interactions, frequency of bacterial resistance development was determined. An exponentially growing culture of strain CFBP 1687 was infected with KIL3 at MOI 1 and after 72 h incubation, an average of 110 phage-resistant bacteria appeared on plate. To exclude the possibility of contamination, the identity of 12 colonies was verified with PCR-analysis using specific primers, confirming their identity as *P. syringae* pv. *porri*. This resulted in a resistance frequency of 1.83×10^{-6}. A host screen with those resistant bacterial derivatives revealed that five of them were resistant to the six phages, a phenomenon called cross-resistance. The other isolates could still be infected by the host-range mutant phage KIL3b. The same experiment was performed with phage KIL4. After 72 h incubation on average 200 resistant colonies appeared and PCR-analysis was used to confirm the identity of 12 of them, resulting in a calculated resistance frequency of 3.33×10^{-6}. Again, cross-resistance was observed for part of the strains (four out of 12), the other resistant strains were still infected by KIL3b.

A spot test with supernatant from an overnight culture of those resistant isolates on strains CFBP 1687 and LMG 28496 suggested there was no induction of lysogens under the conditions tested. In general, as all phages produced clear plaques there was no indication for lysogeny.

Genome and Proteome Analysis

Genome Analysis

The genomes of all six phages were sequenced using the Illumina MiSeq platform and single contigs were obtained. Information about the genome characteristics of the phages is summarized in **Table 2**.

ORFs were identified and similarity at the protein level was verified by Blastp analysis. In addition, a putative function could be assigned to some ORFs without homologs using PHYRE2. All tRNAs were found in the same region spanning 2.7 kb in front of the large terminase subunit, a part of the packaging machinery (**Figure 2**). This is consistent with the genome organization of the related PAK_P1-like phages (Debarbieux et al., 2010; Henry et al., 2015). When comparing the tRNAs present in the six genomes, phages KIL1, KIL3, and KIL3b encode the same five tRNAs. KIL2, KIL4, and KIL5 also have these five tRNAs in their genomes, supplemented with four additional tRNAs. Depending on the phage, 17 or 18 rho-independent terminators were retained after manual verification and were located across the entire genome. Probable promoter sequences were identified by looking for conserved motifs in the 100 bp upstream region of every ORF. Using MEME, a motif resembling a typical *E. coli* bacterial promoter sequence (TTGACA-N_{17-18}-TATAAT) was found in all phages. As in the most closely related *P. syringae* phage PhiPsa374, the 41 bp conserved motif surrounding the bacterial promotor sequences in PAK_P1-like viruses was not encountered thereby confirming its specificity for *P. aeruginosa* phages (Henry et al., 2015). No toxin genes, virulence genes or genes related to lysogeny were discovered in the phage genomes, indicating their suitability for phage therapy.

A remarkable feature of all KIL-like phages is the presence of a homopolymeric G-stretch of variable length at one or two locations in the genome (Table S1). Illumina reads at those positions contain 6–13 Gs. Both stretches are found in intergenic regions, yet not clearly associated to known promoter elements. A similar feature was reported previously in PB1-like phages, in a sequence coding for a baseplate protein and in the ORF coding for a DNA adenine methylase in Bordetella phages BPP-1, BIP-1, and BMP-1 (Liu et al., 2004; Ceyssens et al., 2009). It was proposed that the resulting frameshift serves as a control point for expression levels of the protein. Another remarkable region in the KIL-like phage genomes is a long A-stretch varying from 14 to 33 nucleic acids in length. In the most related phage phiPsa374 those homopolymeric stretches are not present. A phylogenetic analysis of the six phages based on their genome sequences confirms the subdivision of the phages into two groups, correlated to their host range. The first group constitutes of phage KIL1, KIL2, KIL3, and KIL3b, the second group contains phages KIL4 and KIL5. Between the groups, some differences in the ORFs are predicted, mostly among unknown genes. The difference between the genomes of phage KIL3 and KIL3b, which is a host range mutant of the former, is limited to the number of repeats located in the homopolymeric A- and G-stretches. No clear biological function could be attributed to this phenomenon.

The Phylogenetic and Proteome-Based Relationships

To further frame these phages in their comparative genomic context, phylogenetic trees were constructed based on the concatenated datasets of two structural genes encoding the major capsid protein and the baseplate protein. These two proteins

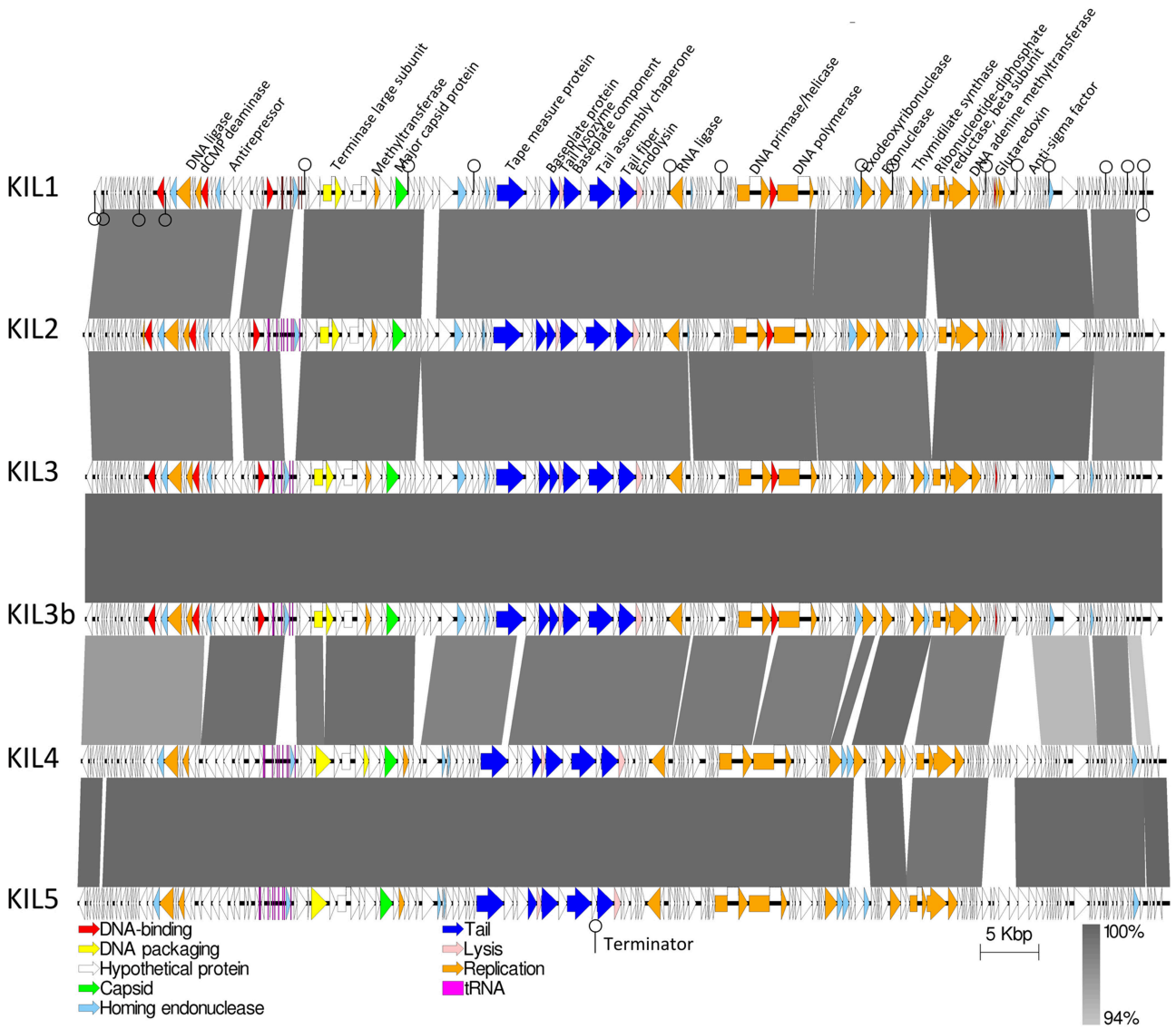

FIGURE 2 | Representation of the genome organization of the six KIL-like phages and homology (blastn) between them.

are shared among the genomes of four clades: the FelixO1-like viruses, PAK_P1-like viruses, and KPP10-like viruses within the *Felixounalikevirus* genus and the rV5-like viruses, as well as phages CR3, ICP1, and our six phage isolates. Each protein was assigned to "Family:virus:314" and "Family:virus:3622" by ACLAME-based clustering (Table S2). Phylogenetic analysis revealed the four clades comprising the KPP10-, PAK_P1-, FelixO1, and rV5-like phages, respectively (Left, **Figure 3**). Most clades branch deeply and are well supported with bootstrap support values of more than 90%. Within the *Felixounalikevirus* genus, the *Pseudomonas* phages KIL1, KIL2, KIL3, KIL4, KIL5, and KIL3b are grouped into a distinct clade, together with a *Pseudomonas* phage phiPsa374.

To investigate the genome relatedness based on the proportion of conserved homologous proteins between two phages, we performed pairwise comparisons of the 32 phage genomes, which allowed us to assign 5622 protein sequences to 1322 homologous protein families (Table S1). A cut-off value of 40% of homologous proteins was used to assign phage genomes to a putative genus (Lavigne et al., 2008, 2009b). The pattern of proteome-based grouping was highly similar to our phylogenetic results, in which KIL1, KIL2, KIL3, KIL4, KILP5, KIL3b, and phiPsa374 form a conserved group with ≥50% homologous proteins in common to each other's genome (Right, **Figure 3**). These seven phages are also related to three groups of the *Felixounalikevirus* phages, sharing more than 20% of their homologous proteins, with the PAK_P1-like phages being the closest relatives (average 34% shared proteins). Notably, of these, phiPsa374 shared the highest overlap percentages of the proteomic content (≥40%) with the PAK_P1-like phages.

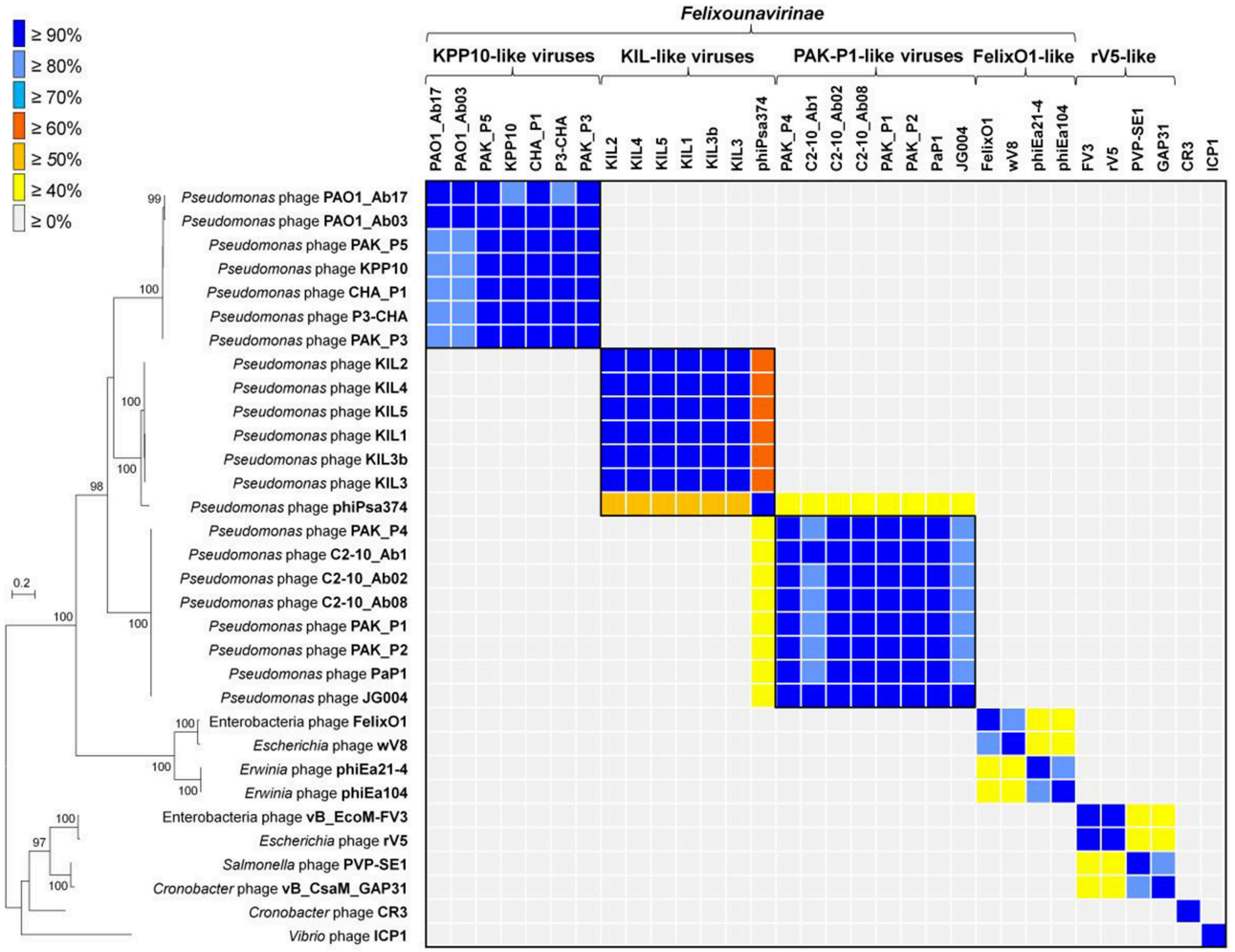

FIGURE 3 | Phylogenetic and proteome-based relationships of phages KIL1, KIL2, KIL3, KIL4, KIL5, and KIL3b. Left, maximum-likelihood tree was constructed upon a concatenation of two structural proteins (major capsid protein and baseplate protein) that are common to the 32 phages. The numbers at the branch represent the bootstrap values. Edges with bootstrap values above 75% are represented. The scale bar indicates the number of substitution per site. Right, in the matrix view, the columns and rows correspond to the phages. Each cell indicates the percentage of shared protein families between two phages. Color tags are used to visualize the genomic relatedness based on the proportion of shared protein families.

Proteome Analysis

Analysis of the proteome of phages KIL3 and KIL5 (representatives of the two clades) by ESI-MS/MS led to the identification of 51 and 67 proteins, respectively (Table S3). For KIL3 and KIL5, respectively 27 proteins and 26 had a predicted function based on homology to other phage proteins. In both phages, most of the detected proteins are early phage proteins or structural phage proteins. The use of non CsCl-purified phages might explain the presence of the non-structural phage proteins since they can remain in the phage lysate after bacterial lysis. Alternately, these proteins may be considered as candidate proteins which may be co-injected during infection. The most abundant proteins based on the number of unique peptides recovered are the tape measure protein, ORF 46 (no predicted function) and the major capsid protein in phage KIL3. Similarly, the most abundant proteins of phage KIL5 are the capsid protein, ORF 46 (no predicted function) and the tail fiber protein (ORF 76). As was seen by Henry et al. (2015) for the related phage PAK_P3, the ORF upstream of the capsid protein had the second highest relative abundance (total number of spectra / Molecular Weight) next to the capsid protein itself, and this in both phages. Although the function of this protein could not be predicted, an association with the capsid protein is suspected. The differences in the number of proteins identified for the two phages could be explained by a difference in protein concentration as the number of peptides recovered for proteins of KIL5 were in general higher than those for KIL3. The identification of these peptides by mass spectrometry confirms the *in silico* ORF predictions. One structural protein, a tail lysozyme (ORF 70), could not be detected in KIL3; however, the corresponding protein in KIL5 (ORF 71) was identified. All other predicted structural proteins could be experimentally confirmed by mass spectrometry.

Phage Therapy

Bio-Assay on Leek Leaves

To investigate whether the phages were also capable of lysing their bacterial host in the plant environment, bio-assays were performed on leek leaves.

In a first bio-assay, the bacterial concentration was chosen to be 10^7 CFU/ml, the amount of bacteria necessary to produce clear symptoms. For the phages, a relatively high concentration of 10^9 pfu/ml was used to prove efficacy. All 41 *P. syringae* pv. *porri* strains were inoculated and showed comparable virulence, presented on the left side of the boxplot (**Figure 4**). Strains CFBP 1687 and CFBP 1770 were chosen as representatives of *P. syringae* pv. *porri* and were used as bacterial host in combination with the different phages. Lesion lengths on the phage treated leaves were compared to the lesion lengths induced by all *P. syringae* pv. *porri* strains used in this study. In general, each phage reduced the mean lesion length compared to the non-phage treated leaves. The reduction in lesion length was significant for phages KIL1, KIL3, and KIL3b with *p*-values of 0.003, 0.005, and 0.004, respectively. Phages KIL2, KIL4, and KIL5 reduced the lesion length, but were not able to prevent bacterial infection completely.

In a second bio-assay, the effect of the phages on a lower bacterial concentration of 10^6 CFU/ml was tested using ten leaves per phage. CFBP 1687 was used in combination with phages KIL1, KIL2, KIL3, and KIL3b, phages KIL4 and KIL5 were co-inoculated with bacterial strain LMG 28496. Again, lesion lengths of the phage-treated leaves were compared to the lengths produced by their respective host without phage. As expected, lesion lengths were smaller in all treatments compared to the previous assay with higher bacterial concentrations. Only phages KIL2 and KIL3b decreased lesion length significantly compared to their bacterial control, with *p*-values of respectively 0.042 and 0.006. Leaves treated with phage KIL3b showed no symptoms for the most part. The results of this second bio-assay confirm that phages KIL4 and KIL5 are less suitable to reduce lesion length significantly. Only phage KIL3b showed significant reduction in both assays, demonstrating its *in planta* antibacterial effect. The effect of phages KIL1, KIL2, and KIL3 varied between the assays. Nevertheless, it was considered prudent to maintain all phages in a cocktail for subsequent testing in field trials.

Field Trials

In a first field trial set-up, the potential of phages to protect leek transplants against bacterial infection at planting was tested. Transplants were submerged in a phage solution containing the six phages before planting them in an infested field. This experiment was performed on three different locations in Flanders in the year 2014 (**Table 3**).

In the trial at PCG, leek plants of cultivar Kenton (Nunhems) were used. The first symptoms developed 25 days after planting in phage-treated and non-treated plants. Two months later, percentages of diseased plants increased to 82% for the phage-treated plants and 92.5% for the control plants. Later on, symptoms disappeared again in both groups and percentages of diseased plants were again lower 4 months after planting. The same trend was apparent when scoring the percentage of leaf surface affected.

The same experiment was performed in PSKW with cultivar Harston (Nunhems). Two and three months after planting, the disease incidence was measured. Percentages of symptomatic plants varied from 29% for the phage-treated plants and 19% for the control plants after 2 months to 18 and 15% after 3 months, respectively. No significant differences were observed.

In the experiment at Inagro, leek plants of cultivar Harston (Nunhems) were used. No disease incidence was recorded 2 months after planting. After 3 months, measurements reported an infection rate of 23.5% of control plants and 18% for the phage treated plants.

In all three experiments, no significant differences in disease occurrence between treated and untreated plants could be noticed. In the trials performed at PSKW and Inagro, disease incidence remained low indicating that infection was not successful. Conclusions on the efficacy of phage treatment could therefore not be made. From the trial results of PCG we can conclude that phage treatment was not successful in protecting the leek plants from infection when planted in an infested field. However, monitoring at an earlier time-point could be better to detect an effect of the phages. It is also possible that more frequent phage applications are necessary to protect the plants against infection.

In a second field trial, phages were sprayed on artificially infected plants, to test for their potential as a crop protection agent in a later growth stage. First, bacteria were sprayed onto the plants 3 months after planting. The day after, a phage cocktail with the six phages present in a concentration of 10^9 pfu/ml each was sprayed onto the plants. Results are shown in **Table 4**.

In the trial at PCG, again cultivar Kenton (Nunhems) was used and phage treatment was repeated four times every 2 weeks. The first disease assessment was performed 19 days after infection and demonstrated a higher infection rate at the bacteria treated plant in relation to the other plants, meaning that infection was successful. One month after infection, infection rate in the bacteria-treated plants increased and a difference trend could be measured for the phage-treated object in comparison with the non-treated infected object with, respectively, 38.5 and 63% symptomatic plants. In subsequent measurements this trend continued although the differences became less apparent. The same trend was demonstrated by the data displaying the affected leaf surface. At all time-points, infection was slightly higher in the phage-treated control compared to the untreated plants. The trial demonstrated that phages could decrease symptom development but were not able to completely stop infection. A higher phage concentration and more frequent applications can possibly lead to a better disease control.

The same trial was performed at PSKW with plants of the cultivar Harston (Nunhems). Phage treatments were applied every week for 8 weeks with half the dose of phages compared to the trial of Kruishoutem, meaning that a concentration of 0.5×10^9 pfu/ml of each phage was used. Disease incidence was measured one, 2 and 3 months after infection but no differences between the plants could be measured. Even the untreated and infected plants showed the same level of symptoms, indicating

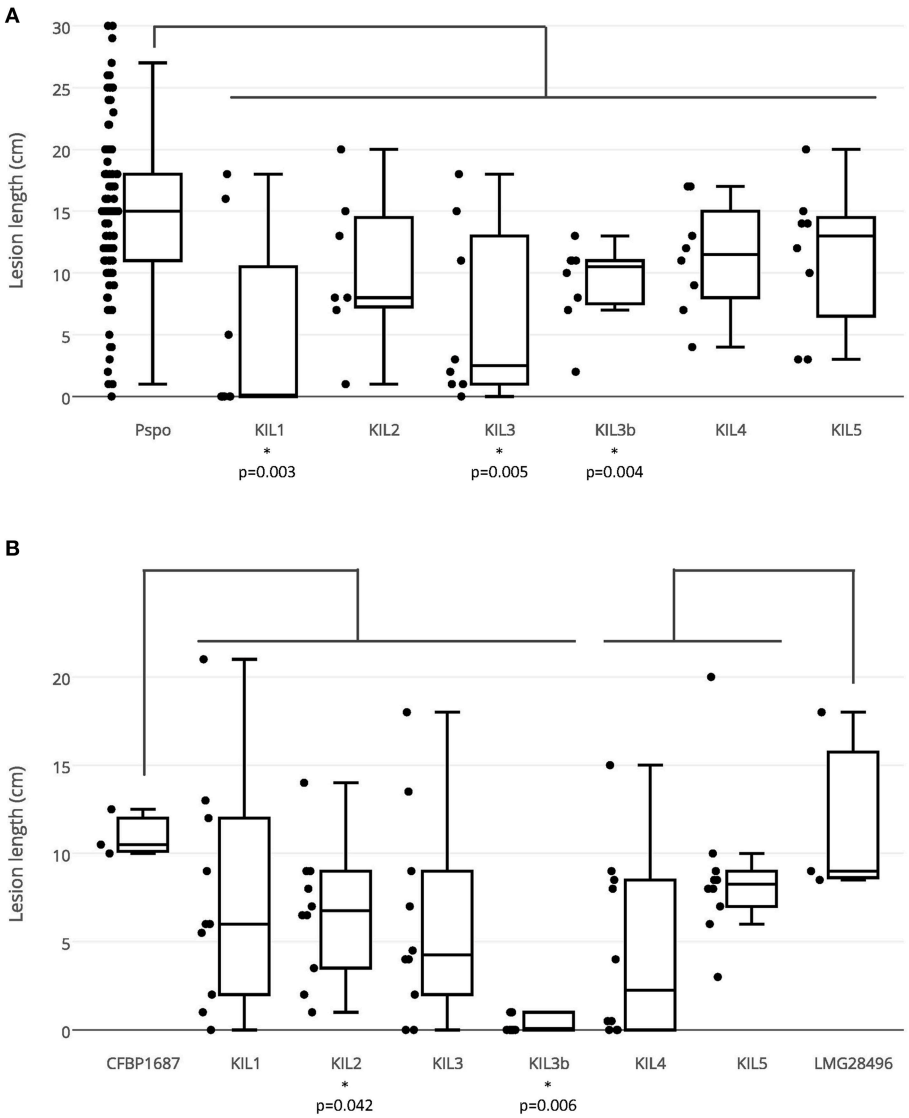

FIGURE 4 | (A) Lesion lengths in the first bio-assay in which all 41 *P. syringae* pv. *porri* strains (10^7 CFU/ml) were inoculated in leek leaves (three inoculated leaves/strain) and all phage types (10^9 pfu/ml) were co-inoculated with a *P. syringae* pv. *porri* representative (eight inoculations/phage-bacteria combination). **(B)** Lesion lengths in the second bio-assay in which KIL1, KIL2, KIL3, and KIL3b (10^9 pfu/ml) were co-inoculated with bacterial strain CFBP 1687 (10^6 CFU/ml) and phages KIL4 and KIL5 with LMG 28496 (10 inoculations/phage-bacteria combination).

TABLE 3 | Percentage symptomatic plants after 3 months for the field trial at three different locations where 1200 transplants were submerged in phage solution (10^7 pfu/ml) before planting in a field infested with CFBP 1687.

Phage treatment	PCG	PSKW	Inagro
No	92.5	15.0	23.5
Yes	82.0	18.0	18.0

the possibility that symptoms were masked by natural infection with another pathogen.

The trial performed at Inagro used plants of the cultivar Harston (Nunhems). Five phage applications were carried out: one a week before infection, one the evening of the day during which bacterial infection was performed, and three more weekly applications after infection. Two months after infection disease incidence was measured showing no differences between the four treatments. Even the uninfected and untreated control and the uninfected phage-treated control displayed an infection of respectively 19.5 and 24% indicating the presence of a natural infection that could not be affected by our phages.

Results of the three trials demonstrate that only at PCG conducive conditions for infection with *P. syringae* pv. *porri* were present. From that trial we can conclude that phage application can lead to a reduction in bacterial disease incidence but cannot

TABLE 4 | Percentage symptomatic plants after 1 (PCG and PSKW) and 2 months (Inagro) for the field trial with *P. syringae* pv. *porri* CFBP 1687 (10^6 CFU/ml) and the phage cocktail (10^9 pfu/ml) being sprayed over 1000 leek plants per treatment.

Treatment	PCG	PSKW	Inagro
Untreated	6.0	32.0	19.5
Phage	11.0	38.0	24.0
Bacteria	63.0	42.0	21.5
Bacteria + phage	38.5	30.0	19.0

completely prevent it. Other factors such as natural infections with other pathogens make it difficult to predict the effect and reliability of phage therapy in field trials.

DISCUSSION

The increase of bacterial blight of leek and the related economical losses attracted our interest in the problem a few years ago leading to an extensive investigation of the causative agent, *P. syringae* pv. *porri*. Previous research divided the Flemish isolates into two groups based on their BOX-PCR fingerprints (Rombouts et al., 2015). Genomic differences among *P. syringae* pv. *porri* strains were also described by Noble et al. (2006), who showed minor differences based on profiles generated with IS50-primer amplification and RFLP of a 16S rDNA fragment. In contrast, these authors as well as Koike et al. (1999) and van Overbeek et al. (2010) did not discriminate different genotypes among their isolates from California, Australia and the Netherland when applying different repetitive-element-PCRs (BOX, REP, and ERIC). This overall bacterial diversity was kept in mind when looking for novel phages capable of lysing *P. syringae* pv. *porri*. Host range analysis of the five phages isolated from soil and the H-mutant phage confirmed the BOX-PCR grouping of bacterial strains, suggesting that differences at the bacterial genome are responsible for the observed host range. Previous genome analysis of a bacterial strain of each group demonstrated that variation was mostly situated in prophage content and mobile genetic elements (Rombouts et al., 2015). No specific element could be found that linked bacterial diversity with the phage host range. In general, genomic differences can affect phage infection at different stages. If those genome differences result in an altered phage receptor, different receptor binding proteins (RBP) capable of recognizing the altered receptor of the five bacterial strains in group one could be present among the phages KIL4 and KIL5. Changes in host range due to a spontaneous mutation in a tail fiber protein (replacement of a positively charged lysine by an uncharged asparagine) were previously reported for two related phages, *Pseudomonas aeruginosa* phages PaP1 and JG004 (Le et al., 2013). However, the exact role of the variation and mutation observed here does not lead to a straightforward hypothesis and requires further investigation. In spite of the overall bacteriophage diversity, all phages we found against *P. syringae* pv. *porri* belong to the *Myoviridae* family. Genome sequence analysis further classified them into a new clade within the *Felixounavirinae* genus.

The phage characteristics analyzed in this study show that all six phages are suitable for use in phage therapy. First, they are strictly virulent, meaning that infection with the phages results in bacterial lysis minimizing the risk of horizontal transfer of pathogenicity genes as is the risk with lysogenic phages (Penadés et al., 2015). Furthermore, by demonstrating a temperature stability from 4 to 37°C and pH stability from pH 4 to 12, they are capable of surviving in the plant environment when applied. However, a disadvantage is their limitation to infect only bacteria grown at temperatures of 28°C or lower. This means that in summer, when temperatures occasionally rise above 28°C bacteria may not be killed by the phages. Probably, the bacterial receptor necessary for phage infection is not expressed at higher temperatures. Temperature dependent infection was previously reported for phage φS1 which is not able to infect *Pseudomonas fluorescens* cells grown at 37°C (Sillankorva et al., 2004). It has been shown that expression of bacterial genes can be temperature dependent. For example, the expression of flagellar components and the surfactant syringafactin is reduced in *P. syringae* strain B728a at a temperature of 30°C, resulting in reduced swimming and swarming motility (Hockett et al., 2013). Host range analysis demonstrates that none of the phages is capable of lysing all tested *P. syringae* pv. *porri* strains, but a combination of different phages in a phage cocktail can cover all strains used. The results indicate that there is some resistance development among *P. syringae* pv. *porri* strain CFBP 1687 when challenged with the bacteriophages KIL3 or KIL4. Although the kill curves indicate that in the first 5 h there is no resistance development, after 72 h an average resistance frequency of around 2×10^{-6} was noted. When phages are applied in a phage cocktail, this resistance development is less likely to occur (Gill and Hyman, 2010; Barbosa et al., 2013). The possibility of resistance through lysogenic conversion was excluded, thereby confirming their suitability for phage therapy for this parameter. In addition, development of the host-range mutant phage KIL3b demonstrates that host-range expanding adaptions are possible to counter bacterial resistance, especially because this phage was still able to infect more than half of the resistant bacteria.

To investigate evolutionary relationships of KIL1, KIL2, KIL3, KIL4, and KIL5 with their close relatives, phylogenetic analysis was combined with a whole genome proteomic approach (Lavigne et al., 2008, 2009b). The overall phylogenetic relationships between the concatenated sequences of the major capsid protein and the baseplate proteins of the *Felixounalikevirus* phages are consistent with previous taxonomic description (Henry et al., 2015). The phages KIL1, KIL2, KIL3, KIL4, and KIL5 as well as phiPsa374 can clearly be grouped into a new phylogenetic clade. Their location within the *Felixounalikevirus* genus suggests that the sequences of two structural proteins are highly conserved within their own group and appear to be most closely related to those of the "PAK_P1-like viruses" and "KPP1-like viruses." In addition, our proteome-based analysis, which is more sensitive and accurate for taxonomic classification for phage genomes (Lavigne et al., 2008), supports the phylogenetic relationships of the *Felixounalikevirus* phages. Given that shared homologs between the phage genomes can be used as an indication of

phage members in the same genus (Lavigne et al., 2008, 2009b), we proposed a possible division of the *Felixounalikevirus* genus into the four subclades, which include the "KPP10-like viruses" (phages PAO1_Ab17, PAO1_Ab03, PAK_P5, KPP10, CHA_P1, P3-CHA, and PAK_P3), "PAK_P1-like viruses" (PAK_P4, C2-10_Ab1, C2-10_Ab02, C2-10_Ab-8, PAK_P1, PaP1, and JG004), "FelixO1-like viruses" (FelixO1, wV8, phiEa21-4, and phiEa104), and "KIL-like viruses" (KIL1, KIL2, KIL3, KIL4, KILP5, KIL3b, and phiPsa374). Interestingly, although phage phiPsa374 has been suggested as a member of the PAK_P1-like clade (Frampton et al., 2014; Henry et al., 2015), our results indicate the genome of phiPsa374 aligns with more shared protein families to the KIL-like phages.

No toxin genes, virulence genes or genes related to lysogeny could be discovered in the phage genomes at this time, indicating their suitability for phage therapy. Efficacy of the phages in the plant environment was analyzed with bio-assays in leek leaves. Significant results were only obtained for phages KIL1, KIL2, KIL3, and KIL3b but the broader host range of phages KIL4 and KIL5 led to their incorporation in the phage cocktail for the field trials. When treating transplants with a phage cocktail before planting in an infested field, no significant differences were observed. Spraying of phages on artificially infected plants led to a slightly reduced symptom development in one of the three trials, yet again without statistical significance. Since two trials suffered from natural infections, their impact has most likely masked the effect of the treatments. Although isolation and characterization of phages is reported for several plant pathogens, results of other field trials are scarce. A recent overview has recently been published by Czajkowski (Czajkowski, 2016). A field trial with phages against *Dickeya solani* on potato also reports of phage therapy resulting in minor differences in disease severity (Adriaenssens et al., 2012). In experiments with phages against *X. axonopodis* pvs *citri* and *citrumelo*, significant control of bacterial spot was reported, yet treatment with copper-mancozeb was more effective (Balogh et al., 2008). As other researchers mentioned, open field (and greenhouse) applications with phages encounter many challenges such as UV-light, desiccation, application method, and the need of a large quantities of phages. Especially the phyllosphere is a harsh environment for phages, making leaf diseases difficult to treat (Gill and Abedon, 2003; Goodridge, 2004; Iriarte et al., 2012). This study indicates that phage therapy is able to reduce bacterial blight symptoms in leek, but to improve the efficacy of the phages in the field, phage persistence in the plant phyllosphere should be improved by the use of protective formulations, addition of non-pathogenic phage-propagating bacterial strains or adapting timing and frequency of application as suggested previously (Jones et al., 2007; Balogh et al., 2010).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: SR, AV, JV, MM, RL. Performed the experiments: SR, AV, SV, BD, DV, CA, CV, HJ, YB, JN, JK. Analyzed the data: SR, DV, YB, MM, RL. Contributed reagents/materials/analysis tools: JN, JK, MM, RL. Wrote the paper: SR. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00279

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Prophylactic Bacteriophage Administration More Effective than Post-infection Administration in Reducing *Salmonella enterica* serovar Enteritidis Shedding in Quail

Mosab Ahmadi[1], M. Amir Karimi Torshizi[1], Shaban Rahimi[1] and John J. Dennehy[2]*

[1] Department of Poultry Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran, [2] Biology Department, Queens College and The Graduate Center of the City University of New York, New York, NY, USA

Infections caused by *Salmonella* bacteria, often through poultry products, are a serious public health issue. Because of drawbacks associated with antibiotic prophylaxis, alternative treatments are sought. Bacterial viruses (bacteriophages) may provide an effective alternative, but concerns remain with respect to bacteriophage stability and effectiveness. To this end, we assessed the stability of a novel bacteriophage isolated from poultry excreta, siphovirus PSE, and its effectiveness in reducing *Salmonella enterica* serovar Enteritidis colonization *in vitro* and *in vivo*. Moreover, we sought to determine how the timing (prophylactic or therapeutic) and route (oral gavage or vent lip) of PSE administration impacted its effectiveness. Here we report that significant quantities of viable PSE bacteriophages were recovered following exposure to high and low pH, high temperatures, and bile salts, testifying to its ability to survive extreme conditions. In addition, we found that ileal lactic acid bacteria and *Streptococcus* spp. counts increased, but colibacilli and total aerobe counts decreased, in quail receiving phage PSE through both oral gavage and vent lip routes. In other experiments, we assessed the efficiency of PSE administration, in both prophylactic and therapeutic contexts, via either oral gavage or vent lip administration, on *S.* Enteritidis colonization of quail cecal tonsils. Our results demonstrate that administration of PSE as a preventive agent could reduce the *S.* Enteritidis colonization more effectively than post-challenge administration. Furthermore, oral administration of PSE phage is a more effective prophylactic tool for reduction of *S.* Enteritidis shedding in poultry than is vent lip administration.

Keywords: phage therapy, prophylactic agent, *Salmonella* Enteritidis, phage administration method, microflora population

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****Correspondence:***
M. Amir Karimi Torshizi
karimitm@modares.ac.ir

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INTRODUCTION

Despite impressive advances in the control of infectious diseases, some bacterial pathogens have acquired antibiotic resistance and are emerging in human populations. Many of these infections are zoonotic and are transmitted from healthy carrier animals to humans through contaminated food (Wegener et al., 2003). For example, *Salmonella* bacteria, especially the serovars Typhimurium and

Enteritidis, are common contaminants of poultry and eggs, causing food-borne disease and death (Borie et al., 2008). In order to reduce pathogen contamination of the food chain and eliminate food poisoning in human population, the eradication of *Salmonella* infections before harvest and processing is crucial (Seo et al., 2000; Mølbak and Neimann, 2002). Efforts to control *S.* Enteritidis in poultry historically have relied on a combination of farm biosecurity and the prophylactic application of antibiotics, but these strategies are increasingly unreliable (Hugas and Tsigarida, 2008; Buncic and Sofos, 2012). First, indiscriminate use of antibiotics has been implicated in a surge in multidrug resistant *S.* Enteritidis. Second, poultry consumers are increasingly concerned that edible poultry tissues are contaminated with harmful concentrations of drug residues (Donoghue, 2003; Griggs and Jacob, 2005). Third, changes in food production, food rejection, and preventive measures have incurred significant economic losses to poultry producers (Tsonos et al., 2013).

Furthermore, most antibiotics, up to 90% of orally administered doses, are not fully absorbed in the chicken gut, and can be excreted in the feces unchanged (Kumar et al., 2005). As one of the largest food-producing industries in the world, the poultry industry generates enormous amounts of manure and litter, much of which is currently applied to agricultural land (Bolan et al., 2010). The effects of antibiotics contained in manure on soil microbial communities are largely unknown (Ollivier et al., 2010). In addition, poultry litter from contaminated farms may introduce zoonotic pathogens such as *S.* Enteritidis into the environment (Line and Bailey, 2006). Thus the application of poultry litter as fertilizer can create significant public and environmental health concerns. Because of these issues, there is an urgent need to find novel and effective *S.* Enteritidis control strategies to minimize the risk of spreading antimicrobial resistance among animal and human populations.

Ideally vaccination would be an optimal control strategy, but *S.* Enteritidis vaccines do not provide complete protection to infected chickens (Gast et al., 1992). There are more than 2,500 serovars of *Salmonella*, and vaccines made from any one serovar often do not confer cross-protection against others (Singh, 2009). An old strategy of using naturally occurring bacterial viruses (bacteriophages) to tackle infections, is regaining popularity (Tsonos et al., 2013). The therapeutic and prophylactic application of bacteriophages is generally considered safe (Johnson et al., 2008). Bacteriophages are highly specific to certain bacterial strains, thus presumably have minimal impact on natural human or animal microflora populations (Sulakvelidze et al., 2001). However, bacteriophage therapy has not been consistently effective (Lu and Koeris, 2011). Issues remain in terms of effective delivery routes, host resistance, limited generalizability, and possible interactions with the immune system (Loc-Carrillo and Abedon, 2011). As capsid-based entities, bacteriophages are especially sensitive to environmental conditions, such as that in the upper gastrointestinal tract. Therefore, oral administration of phages may be limited in efficiency unless the selected phages are especially resilient to environmental challenges.

An alternative method of phage administration, the vent lip of the avian cloaca, was previously described by Andreatti Filho et al. (2007). However, the relative efficacy of oral versus vent lip administration has not been comparatively assessed. In this study, a novel lytic bacteriophage against *S.* Enteritidis, PSE, was isolated and characterized in terms of pH and thermal stability, bile salts tolerance, morphology, host range, and one-step growth. In addition, the therapeutic and prophylactic effects of PSE administration on the bacterial load of quail ilea were compared. Finally, in order to provide additional insight in the feasibility of phage application as an antimicrobial agent in poultry industry, the efficacy of PSE oral versus vent lip administration to reduce *S.* Enteritidis shedding in experimentally infected Japanese quail was determined.

MATERIALS AND METHODS

Bacterial Challenge Strain

The *Salmonella* Enteritidis (RITCC 1695) was purchased from the Razi Vaccine and Serum Research Institute (Karaj, Iran). For culture preparation, bacteria were grown in nutrient broth (Merck, Germany) at 37°C overnight. Culture turbidity was adjusted to match McFarland standard 4.

Isolation, Purification and Enumeration of Bacteriophage

Each poultry excreta sample was collected and suspended 1:10 (w/v) in SM (salt of magnesium) buffer (50 mM Tris-HCl [pH 7.5], 0.10 M NaCl, 8 mM $MgSO4{\cdot}7H_2O$, 0.01% gelatin [Sigma, Germany]), then suspension was centrifuged at 15,610 × *g* for 20 min at 4°C. The resulting supernatant was filtered through a 0.22 μm membrane filter (Jet Biofil, China). Two hundred microliter of each filtered sample was mixed with 50 μl of fresh log-phase *S.* Enteritidis and 7 ml of top agar (0.7% agar in nutrient broth) and poured over a petri dish containing nutrient agar (1.5% agar; Serva, Germany) and incubated at 37°C for 24 h. Individual clear plaques were extracted from the agar overlay with a sterile Pasteur pipette, and suspended in 500 μl of SM buffer, which was then re-plated to obtain single plaques. Each isolate was plaque purified 4x to ensure that each isolate represented a single clone.

Phage stocks were serially diluted in SM buffer to achieve a concentration that would produce discrete plaques on a bacterial lawn. Aliquots of 200 μl of any 10 × dilution series, 50 μl of fresh log-phase *S.* Enteritidis and 7 ml of soft agar (0.7% agar in nutrient broth, 42–45°C) mixed and poured onto a plate containing nutrient agar (Serva, Germany). Plates were incubated for 24 h at 37°C and phage plaques were enumerated.

Phage PSE Lytic Spectrum

To evaluate the lytic spectrum of isolated bacteriophages, the sensitivity of 15 bacterial strains including three *Salmonella* serovars, four *Escherichia coli* serotypes, one *Campylobacter* serotype and seven gram-positive bacteria to isolated bacteriophages was determined by spot plating phage lysates

on bacterial lawns (Adams, 1959). The resulting plates were incubated overnight at 37°C, and subsequently checked for the phage plaque formation on the bacterial lawns.

One-Step Growth

An overnight culture of *S.* Enteritidis (1 ml) was inoculated into fresh medium (100 ml) and incubated at 37°C for 1 h to yield a cell density of 1.5×10^8 cfu ml^{-1}. To this culture, 1 ml of isolated phage was added, giving an approximate multiplicity of infection of 0.83. Samples were taken at 5 min intervals and immediately chilled until diluting and plating for phage quantitation. Viable bacteria were counted before bacteria and phage were mixed, and subsequently assessed at intervals. Latent period was defined as the time interval between the adsorption and the beginning of the first burst, as indicated by the initial rise in bacteriophage titer. Burst size was estimated from triplicate experiments using the equation described by Jiang et al. (1998).

Thermal and pH Stability of Bacteriophage

In order to evaluate the stability of isolated bacteriophage at various temperatures, test tubes containing bacteriophage were placed in a water bath at each temperature (30, 40, 50, 60, 70, 80, and 90°C) for 30 and 60 min (Bao et al., 2011). The surviving phages were diluted and enumerated immediately using the methods described above. In addition, the stability of bacteriophage at different pH was assessed according to the method described by Verma et al. (2009). The pH of nutrient broth was adjusted with either 1 M HCl or 1 M NaOH to obtain solutions with pH ranging from 2 to 11. A total of 1 ml of phage suspension at a titer of 10^{12} pfu ml^{-1} was inoculated into 9 ml of pH-adjusted medium to obtain a final concentration of 10^{11} pfu ml^{-1}. After incubation for 2 h at 37°C, the surviving phages were diluted and counted by the methods described above. These experiments were performed three times, and the results are reported as the mean of three observation ± standard deviation.

Bile Salts Tolerance

To determine bile salts tolerance, 1 ml of bacteriophage (10^{12} pfu ml^{-1}) was placed into 9 ml of nutrient broth supplemented with 0 (control), 0.15, 0.3 and 1.0% W/V of bile salts (Quelab, Canada). After 0.5, 1, 3, and 4 h of incubation at 37°C, phage survival was assayed by diluting and enumeration of phage using the methods described above. Phage titer in each concentration of bile salts was compared with a bile salt-free control.

Phage Morphology Examination

A drop of 10^9 pfu ml^{-1} bacteriophage was spotted onto a formvar-coated grid and fixed for 2 min with 2.5% glutaraldehyde. Excess sample was removed, and the grid was washed with a drop of double-distilled water. Negative staining was performed by adding 1 drop of 2% uranyl acetate to the grid surface, and excess stain was removed immediately. The grid was allowed to air dry for 60 min and was then observed with a Zeiss-EM 10C transmission electron microscope (TEM) at 100 kV. Phage morphology and dimensions (head diameter, tail length) were determined by the mean of at least 5 measurements.

Analysis of Bacteriophage Nucleic Acid

Extraction of phage nucleic acid was conducted according to the method of Binetti et al. (2005) with slight modifications. Phage particles were precipitated with 4% polyethylene glycol 6000 in 0.2 M NaCl for 48 h at 4°C and centrifuged at 15,610 × *g* for 240 min at 4°C. The pellet was re-suspended in SM buffer, supplemented with 40 μg/ml RNase A (Sinaclon, Iran) or 1 μg/ml DNase I (Sinaclon, Iran) and incubated at 37°C for 30 min. Then 80 μl of lysis solutions (0.25 M EDTA [pH 8.1], 0.5 M Tris-HCl [pH 9.6] and 2.5% sodium dodecyl sulfate [SDS]) were added to 400 μl of a concentrated suspension of phage particles, and the mixture was incubated at 65°C for 30 min. One hundred microliter of 8 M potassium acetate was then added, and the mixture was incubated on ice for 15 min before centrifugation (15,610 × *g*, 30 min, 4°C). Phage nucleic acid was precipitated from the supernatant with an equal volume of isopropanol, kept at room temperature for 5 min, then centrifuged again (15,610 × *g*, 30 min, 4°C). The pellet was re-suspended in 630 μl of TE (Tris EDTA) buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) in the presence of 0.3 M sodium acetate and precipitated with isopropanol for 5 min, followed by centrifugation (15,610 × *g*, 30 min, 4°C). The pellet of nucleic acid was washed twice with 70% ice-cold ethanol, dried and re-suspended in 50 μl of TE buffer.

Purified nucleic acid was digested with DNase I or RNase A according to the manufacturer's instructions to determine nucleic acid type. We also attempted digestion with restriction enzymes *Eco*RI and *Hin*dIII according to the manufacturer's instruction (Serva, Germany). Products of digested phage nucleic acid were electrophoresed by 0.8% agarose gel in a TAE (Tris Acetate EDTA) buffer (40 mM Tris-acetate, 1 mM EDTA) and visualized by transillumination with UV light after the gels were stained with ethidium bromide (Serva, Germany).

Determination of Phage Genome Size by Agarose Gel Electrophoresis

The relatively small genome of PSE made it possible to use agarose gel electrophoresis for determination of its size. The distance of PSE DNA migration on agarose gel electrophoresis was compared to that of un-cut and *Hin*dIII lambda DNA fragments as DNA size marker by Photo Capt software version 12.4 (Vilber Lourmat, France) (Brown, 2010).

Experimental Analysis of Bacteriophage Treatment on *S.* Enteritidis Infection

This project maintained proper ethical standards and all experiments were approved by the Animal Care Committee of Tarbiat Modares University.

Experiment 1

To evaluate the effects of isolated phages on bird natural microflora, ninety 33-day-old *Salmonella*-free quail were

randomly divided into three groups of 30 birds. All birds received an identical diet, and feed and water were supplied *ad libitum*. A control group (Group 1) did not receive bacteriophage treatment. Group 2 received 10^9 pfu ml^{-1} bacteriophage in 100 μl aliquot by oral gavage for 2 days, once every 24 h and group 3 received 10 μl of 10^{10} pfu ml^{-1} bacteriophage, via vent lip route for 2 days, once every 24 h.

Bacterial quantitation of the ileal contents of 10 birds per each group was done at 36 days. One gram of the ileal contents of each bird was serially diluted in phosphate-buffered saline from 10^{-1} to 10^{-6}. Dilutions were subsequently plated on selective agar media for enumeration of target bacteria. Lactic acid bacteria, colibacilli, streptococci and total aerobes were plated on de Man, Rogosa and Sharpe agar (MRS, Merck, Germany), Mac Conkey agar (Merck, Germany), Kenner Fecal *Streptococcus* agar (KF *Streptococcus*, Merck, Germany), and plate count agar (Merck, Germany), respectively. Plates were incubated at 37°C overnight, and then the resulting colonies were counted.

Experiment 2

To compare the efficiency of prophylactic versus therapeutic application of phage on *S.* Enteritidis shedding, one hundred 8-day-old *Salmonella*-free quail were randomly divided into four groups of 25 birds. Group 1 (prophylactic treatment) received 100 μl of 10^6 pfu ml^{-1} bacteriophage via oral gavage for 3 days, once every 24 h, before being challenged orally by 100 μl of 1.2×10^9 cfu ml^{-1} *S.* Enteritidis (**Table 1**). Group 2 (therapeutic treatment) received 100 μl of 1.2×10^9 cfu ml^{-1} *S.* Enteritidis first, then received bacteriophage as described in group 1 (**Table 1**). Birds of group 3 were challenged like group 2, but did not receive phage treatment (**Table 1**). Neither *Salmonella* nor bacteriophage was administered to group 4 (**Table 1**).

The presence of *S.* Enteritidis was assayed at 12, 24 h, and 7 days following *S.* Enteritidis challenge. Birds were euthanized by CO_2 inhalation, and their cecal tonsils were aseptically removed, homogenized and incubated overnight at 37°C in 10 ml of peptone water buffer (Merck, Germany). Subsequently, 100 μl of cultured peptone water was transferred into Rappaport–Vassiliadis broth (Merck, Germany) at 37°C for 24 h, and then streaked on xylose-lysine desoxycholate (XLD) agar plates (Merck, Germany). Plates were incubated at 37°C for 24 h. Any black colonies that formed were identified as *Salmonella* Enteritidis. Any doubtful colonies were plated on triple sugar iron agar (TSI agar, Merck, Germany) slants. In addition, urease activity was checked by assaying the degradation of urea in urea broth (Stager et al., 1983). The recovery of *S.* Enteritidis is reported as the number of *S.* Enteritidis positive samples per number of total samples.

Experiment 3

The effectiveness of oral gavage versus vent lip administration of phage on *S.* Enteritidis colonization in cecal tonsils of quail was evaluated in a third experiment. Three hundred, 1-day-old *Salmonella*-free quail were randomly assigned to five groups of 60 birds. Each group of birds was placed in cages in a controlled environment under strict biosecure conditions. Fecal samples were taken to determine if any pre-existing *Salmonella* phages were present using the method described above. Feed and water were provided *ad libitum*, and birds were maintained at an age-appropriate temperature for the duration of the experiment.

Neither *Salmonella* nor bacteriophages were administered in group 1. Group 2 birds did not receive bacteriophage, but at the age of 4 days were challenged orally by 100 μl of 1.2×10^9 cfu ml^{-1} *S.* Enteritidis. Birds in group 3 received 100 μl of 10^9 pfu ml^{-1} bacteriophage via oral gavage every 24 h for 6 days, starting 3 days before *S.* Enteritidis challenge. Group 4 received 5 μl of 2×10^{10} pfu ml^{-1} bacteriophage, via the vent lip route every 24 h for 6 days, starting 3 days before *S.* Enteritidis challenge. Birds of group 5 received bacteriophages similar to groups 3 and 4, but at half the concentration, and were not challenged with *S.* Enteritidis (**Table 2**). Necropsies of birds from each group were performed at 6, 12 h, 1, 2, 3, 7, 14, 28, and 35 days post-challenge. The presence of *S.* Enteritidis was assayed as described above. In this experiment, recovery of bacteriophage from bird feces was done by using described method for phage isolation.

Statistical Analysis

The data related to the bacterial enumeration of quail ileal contents were compared between groups using a generalized linear model (GLM) implemented in SAS with subsequent Duncan's multiple range test (SAS Institute, 2003). The significance of differences in the incidence of *S.* Enteritidis recovery between control and phage-treated experimental groups was determined by the chi-square test ($p \leq 0.05$).

TABLE 1 | Scheme of Experiment 2.

Day[a]	Group 1 (preventive)		Group 2 (therapeutic)		Group 3 (positive)	Group 4 (negative)
	Phage dose[b]	SE dose[c]	Phage dose[b]	SE dose[c]	SE dose[c]	–
8	10^6	–	–	1.2×10^9	1.2×10^9	–
9	10^6	–	10^6	–	–	–
10	10^6	–	10^6	–	–	–
11	–	1.2×10^9	10^6	–	–	–

[a]*Day of hatching is given as day 0.* [b]*Dose of phage given as pfu ml^{-1}.* [c]*Dose of S. Enteritidis given as cfu ml^{-1}.*

TABLE 2 | Scheme of Experiment 3.

Day[a]	Group 1 (negative control)	Group 2 (positive control)	Group 3 (oral gavage)		Group 4 (vent lip)		Group 5 (phage control)	
		SE dose[c]	Phage dose[b]	SE dose[c]	Phage dose[b]	SE dose[c]	Oral	Vent
1	–	–	10^9	–	2×10^{10}	–	10^9	2×10^{10}
2	–	–	10^9	–	2×10^{10}	–	10^9	2×10^{10}
3	–	–	10^9	–	2×10^{10}	–	10^9	2×10^{10}
4	–	1.2×10^9	10^9	1.2×10^9	2×10^{10}	1.2×10^9	10^9	2×10^{10}
5	–	–	10^9	–	2×10^{10}	–	10^9	2×10^{10}
6	–	–	10^9	–	2×10^{10}	–	10^9	2×10^{10}

[a] *Day of hatching is given as day 0.* [b] *Dose of phage given as pfu ml*$^{-1}$. [c]*Dose of S. Enteritidis given as cfu ml*$^{-1}$.

RESULTS

Phage PSE Isolation and Lytic Spectrum Assay

One *Salmonella*-lysing phage, named PSE, was isolated from poultry's excreta using an enrichment strategy (Cross et al., 2015). PSE forms clear 2–3 mm plaques on *Salmonella* Enteritidis (**Figure 1A**). A lytic spectrum test indicated that PSE was able to lyse three strains of *Salmonellae,* but was unable to lyse bacteria of other genera (**Table 3**).

Phage PSE Morphology

Phage PSE has a round head with a diameter of 51.29 ± 3.15 nm and a tail with length of 74.53 ± 0.74 nm. From its morphology, PSE was presumptively identified as a member of the *Siphoviridae* family with long non-contractile tail in the order of *Caudovirales* (**Figures 1B,C**).

Phage PSE One-Step Growth Dynamics

One-step growth curves of PSE were conducted to determine the growth pattern and the number of progeny phages released

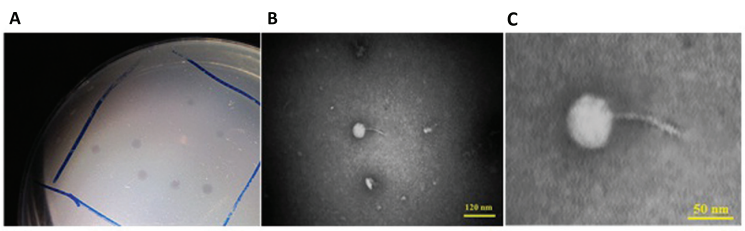

FIGURE 1 | Associated plaque (A) and transmission electron micrographs of PSE phage under 100 kV magnification. The bars represent 120 nm **(B)** and 50 nm **(C)**.

TABLE 3 | The lytic spectrum of PSE on 15 bacterial strains from 7 genera.

Strain	Sources/Reference	Lysis by bacteriophage PSE
Salmonella Enteritidis RITCC 1695	RVSRI[1]	+
Salmonella Typhimurium	Faculty of Veterinary, Tehran University	+
Salmonella Pullorum RITCC 1818	RVSRI[1]	+
Campylobacter jejuni RITCC 1097	RVSRI[1]	–
Escherichia coli O1:K1	RVSRI[1]	–
Escherichia coli O2:K1	RVSRI[1]	–
Escherichia coli O78:K80	RVSRI[1]	–
Escherichia coli Nissle 1819	Isolated from Mutaflor®, Germany	–
Lactobacillus rhamnosus TMU094	Karimi Torshizi et al., 2008	–
Lactobacillus fermentum TMU121	Karimi Torshizi et al., 2008	–
Pedioccus pentosaceus TMU457	Karimi Torshizi et al., 2008	–
Pediococcus acidilactici	Isolated from Bactocell®, France	–
Enterococcus faecalis ATCC 51299	ATCC[2]	–
Enterococcus faecalis ATCC 19433	ATCC[2]	–
Bacillus subtilis	Isolated from Gallipro®, Denmark	–

[1]*Razi vaccine and serum research institute, Iran.* [2]*American Type Culture Collection, USA.*

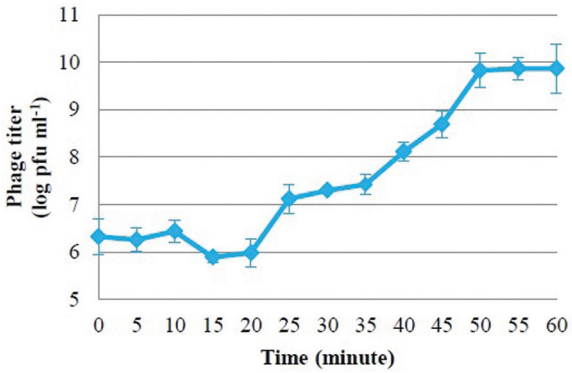

FIGURE 2 | One-step growth curves of bacteriophage PSE in *Salmonella* Enteritidis.

by lysing a single bacterial host cell. One-step growth curves for phage PSE showed a latent period and burst size of about 20 min and 66.66 ± 6.67 pfu cell^{-1}, respectively (**Figure 2**).

Phage PSE Stability at Different Bile Salts Concentrations, Temperatures and pH

The viability of phage PSE was fully maintained and no reduction in PSE titer was observed after 4 h of exposure to any of the tested bile salts concentrations (data not shown). No obvious effect on PSE activity was observed after 2 h incubation at pH levels ranging from 3 to 11 (**Figure 3**). In addition, the results of thermal stability tests suggested that phage PSE was relatively heat stable up to 60 min at temperatures between 30°C to 70 °C. At 80°C or higher, the phage titer quickly dropped, no viral particles were detected after 30 min of incubation, and phage activity was completely lost (**Figure 4**).

Phage PSE Genomic Characterization

The analysis of phage nucleic acid suggested that PSE is a DNA phage as the genome was completely digested by DNase I, but refractory to the activities of RNase A. The estimated genome size of PSE was approximately 35.72 kb (**Figure 5**). Two restriction enzymes used in this study, *Eco*RI and *Hin*dIII, were unable to digest the PSE genome.

Effect of Phage PSE on Bacterial Frequencies in the Quail Ilea

We assessed the impact of phage PSE administration on quail ileal bacteria. Our results show that lactic acid bacteria and streptococci counts increased relative to controls when PSE was administered via oral gavage and vent lip methods (**Table 4**; $P = 0.0001$). By contrast, colibacilli and total aerobes counts decreased relative to controls following both treatment methods (**Table 4**; $P = 0.0001$ and 0.005 respectively). Administration of bacteriophage PSE via oral gavage and vent lip routes increased lactic acid bacteria count by 1.47 and 0.73 log cfu g^{-1}, respectively (**Table 4**). Also streptococci counts increased by 1.05 and 0.58 log cfu g^{-1}, when PSE was administered by oral gavage and vent lip routes, respectively (**Table 4**).

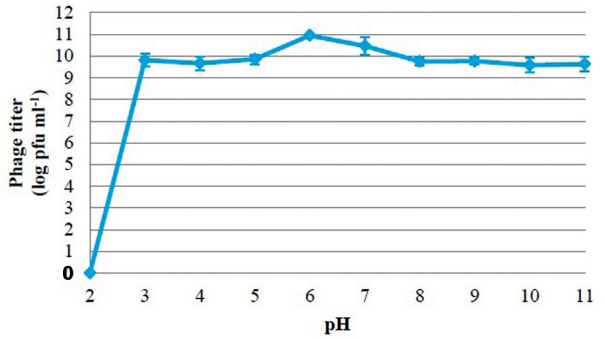

FIGURE 3 | Effect of pH on stability of phage PSE.

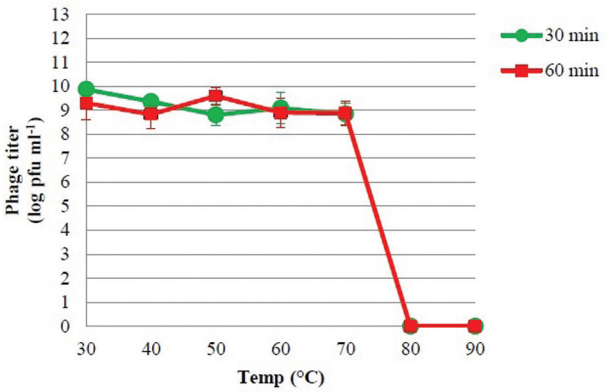

FIGURE 4 | The thermal stability of phage PSE.

Comparison of Preventive versus Therapeutic Phage Treatment

We tested the effectiveness of PSE treatment, either prophylactically prior to *S.* Enteritidis exposure or therapeutically after *S.* Enteritidis exposure. One day post-challenge, *S.* Enteritidis was isolated from 100% of cecal tonsils of positive control and therapeutic groups. By contrast, *S.* Enteritidis isolation rates in birds of negative control and preventive groups were 0 and 33.3% respectively (**Table 5**). In addition, 7 days post-challenge, *S.* Enteritidis was isolated from 100% of positive control and therapeutic groups (**Table 5**). By contrast, the *S.* Enteritidis isolation rate from the preventive group was 20% and birds of the negative control remained free from *S.* Enteritidis.

Comparison of Oral versus Vent Lip Administration of Phage PSE

The application of phage PSE, either by oral or vent lip routes, reduced *S.* Enteritidis recoveries from cecal tonsils of quail in nine consecutive samplings (**Table 6**). Administration of PSE via the oral route was able to eliminate *S.* Enteritidis at all sampling times except 6 h post-challenge. One week after treatment, *S.* Enteritidis was recovered from 100% of cecal tonsil samples of

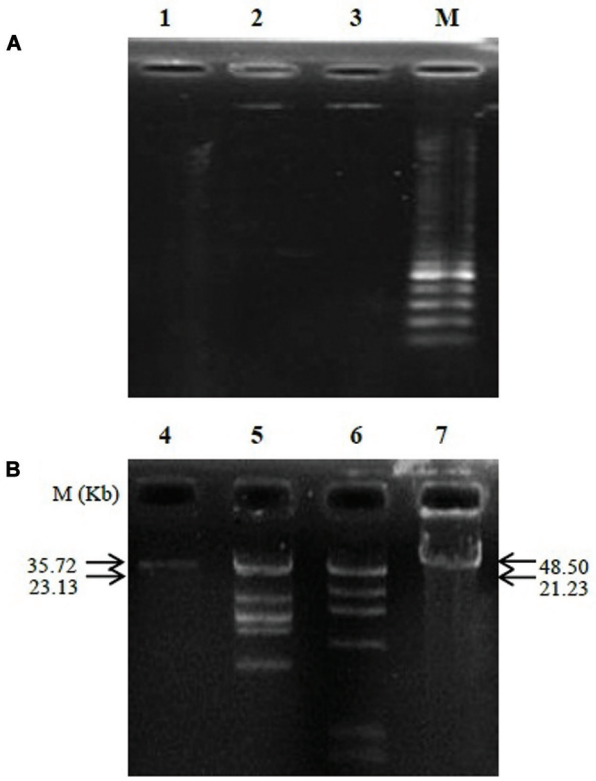

FIGURE 5 | Agarose gel electrophoresis analysis of genomic nucleic digested with DNase I and RNase A (A) and determination of phage genome size (B). Lanes contained the following: 1, digested genome of PSE with DNase I; 2, un-cut genome of PSE with RNase A; 3 and 4, PSE genome; 5, *Hin*dIII + lambda DNA; 6, *Eco*RI + lambda DNA; 7, lambda DNA; M, molecular weight marker.

the positive control group, while no *S.* Enteritidis was found in birds that orally received bacteriophage PSE (**Table 6**). Overall, at all sampling times, *S.* Enteritidis was isolated from 90.74% of cecal tonsil samples of the positive control group. By contrast, *S.* Enteritidis isolation rates in birds treated with bacteriophage PSE via oral and vent lip routes, and in the negative control, were 2.77, 17.58, and 0% respectively (**Table 6**). Bacteriophages were recovered from fecal specimens of all bacteriophage-receiving groups, regardless of administration routes (**Table 7**).

DISCUSSION

A rise in antibiotic-resistant *S.* Enteritidis bacteria, and concerns about the ecological and environmental effects of unrestricted antibiotic usage, have provided strong motivation to find new and effective prophylactic and therapeutic means of eradicating *S.* Enteritidis from poultry. Bacteriophages have excellent potential for replacing or supplementing antibiotics, but their effectiveness and stability must be demonstrated experimentally. Here we describe the isolation, characteristics and effectiveness of a novel bacteriophage able to infect *S.* Enteritidis.

TABLE 4 | Effect of phage PSE treatment on bacterial counts in quail ilea (Log $_{10}$ CFU g^{-1} ileal contents, mean of 10 birds per each group).

Groups	Bacterial group			
	Total aerobes	Colibacilli	Lactobacilli	Streptococci
1 (Control)	8.54	8.31	6.31	5.74
2 (Oral)	7.39	7.99^{ns}	7.78	6.79
3 (Vent lip)	7.79	7.84^{ns}	7.04	6.32
SEM*	0.008	0.01	0.001	0.003
P-value*	0.0001	0.005	0.0001	0.0001

**SEM and P-values were calculated using a GLM univariate analysis of variance. Post hoc comparisons (Duncan's test) of mean values within each column revealed that all are significantly different (P-value < 0.05) from other groups in the same column except the pair labeled with the superscript ns.*

On the assumption that phages isolated from poultry's excreta are more stable in the poultry gastrointestinal tract compared to isolated bacteriophages from other sources, we isolated a bacteriophage, which we called PSE, from poultry excreta. Bacteriophage PSE has a number of characteristics that make it a strong phage therapy candidate. Bacteriophage PSE formed 2–3 mm round, clear plaques on *S.* Enteritidis, implying that it is a lytic phage (Yoon et al., 2007). Because they quickly reproduce within and lyse host bacteria, lytic phages are more suitable than lysogenic phages for phage therapy (Abedon, 2008). Moreover, although PSE was isolated using *S.* Enteritidis, it was also able to infect and lyse *S.* Typhimurium and *S.* Pullorum, two other significant bacterial pathogens found in poultry (**Table 3**).

Bacteriophage PSE's latent period was estimated to be about 20 min, and its burst size was approximately 66.66 ± 6.67 pfu cell^{-1}. There seems to be considerable variation in latent period length and burst size among *Salmonella*-specific phages. Some *Salmonella*-specific phages have been reported to possess small burst sizes and long latent periods, for example ϕ *st*1 (22 pfu cell^{-1}, 40 min) (Wong et al., 2014) and phage Felix 01 (14 pfu cell^{-1}, 60 min) (O'Flynn et al., 2006). On the other hand, some *Salmonella*-specific phages were isolated with burst sizes range from 100 to 200 pfu cell^{-1} and shorter latent periods (Ahiwale et al., 2013). Differences in medium, host cell, pH and temperature may affect variations in latent period and burst size of different phage isolates (Müller-Merbach et al., 2007). For the purposes of phage therapy, optimum latent periods and burst sizes have not been established; however, there is some evidence that these parameters can vary considerably depending on host density and physiological status (Abedon, 1989; Abedon et al., 2001; Wang, 2006).

Phages used for therapeutic or prophylactic purposes should be highly stable and remain viable in a wide range of potential environments. Phage PSE meets some of these requirements. It exhibited powerful antibacterial activity at temperatures ranging from 30°C to 70°C, and pH levels ranging from 3 to 11. Also PSE was found to be resistant to high bile salt concentrations, remaining viable even after 4 h of exposure to 1% bile solution. These characteristics suggest that phage PSE would remain therapeutically viable inside animal bodies, including the animal gastrointestinal tract, where *Salmonella* may be prevalent. All of

TABLE 5 | Frequency of *S.* Enteritidis positive cecal tonsils per group (%).

Groups	Time of sampling		
	12 h[1] post-challenge	24 h[1] post-challenge	7 d[2] post-challenge
1 (Prophylactic)	2/6 (33.33%)	2/6 (33.33%)	2/10 (20%)
2 (Therapeutic)	6/6 (100%)	6/6 (100%)	10/10 (100%)
3 (Positive control)	6/6 (100%)	6/6 (100%)	10/10 (100%)
4 (Negative control)	0/6 (0%)	0/6 (0%)	0/10 (0%)

[1]*Hour.* [2] *Day.*

TABLE 6 | Efficiency of phage PSE administration methods on *S.* Enteritidis recovery from cecal tonsils of Japanese quail (*S.* Enteritidis positive cecal tonsil/ total).

Groups	Time post-challenge								
	6 h[1]	12 h	1 d[2]	2 d	3 d	7 d	14 d	28 d	35 d
1 (Negative)	0/8	0/6	0/8	0/6	0/6	0/6	0/6	0/6	0/8
2 (Positive)	6/8	6/6	6/8	4/6	6/6	6/6	6/6	6/6	8/8
3 (Oral)	2/8	0/6	0/8	0/6	0/6	0/6	0/6	0/6	0/8
4 (Vent lip)	2/8	2/6	0/8	0/6	2/6	0/6	2/6	2/6	0/8
5 (Phage)	0/8	0/6	0/8	0/6	0/6	0/6	0/6	0/6	0/8

[1]*Hour.* [2] *Day.*

TABLE 7 | Recovery of bacteriophage from feces of birds after 7 days post treatment.

Groups	Bacteriophage (Log pfu/g)[a]
1 (Negative control)	0
2 (Positive control)	0
3 (Oral gavage)	10^5
4 (Vent lip)	10^4
5 (Phage control)	10^5

[a]*Average of 10 birds.*

these features point to the utility of phage PSE as a stable agent for control of *S.* Enteritidis colonization of poultry.

In the intestine, bacterial populations can interact with each other, and phages are expected to have a significant role in driving the biodiversity of this complex ecosystem (Ventura et al., 2011). Our data demonstrate that the PSE phage administration, either by oral or vent lip method, strongly affected ileal bacteria frequencies (**Table 4**). We observed lower frequencies of coliforms in phage-treated birds compared with controls (**Table 4**). The declines in coliforms were matched by increases in lactic acid bacteria and streptococci (**Table 4**). *Lactobacillus* spp. are often thought to have a positive effect on poultry health by reducing or preventing colonization of the poultry intestinal tract by undesirable bacteria (Karimi Torshizi et al., 2008). However, given the complexities of the gut microbiome, it is difficult to determine the direct mechanism by which PSE treatment may have influenced the relative numbers of coliforms, lactic acid bacteria and streptococci. This finding is nonetheless an intriguing topic for further investigation.

The *S.* Enteritidis reductions observed in cecal tonsil samples following preventive treatment strongly suggest that phage PSE is a viable prophylactic against *S.* Enteritidis colonization (**Table 5**). It is commonly believed that bacteriophages administered to treated animals are present for the duration of the infection, but once the bacterial host is eliminated so too is the bacteriophage. In our experiments, this did not happen. Bacteriophage PSE was isolated from feces of all groups that received it, even after *S.* Enteritidis infection was no longer observed. Interestingly, phage PSE persistence was also observed in the absence of *S.* Enteritidis challenge (**Table 7**). This finding suggests that phage PSE either remains inactive in gastrointestinal tract for long periods or may have alternate hosts that allow it to proliferate. Similarly, it was reported that, in the absence the primary host, the bacteriophage UZ1 persisted 13 days in a simulated colon (Verthé et al., 2004). This evidence lends credibility to the prophylactic ability of PSE.

This result is in sharp contrast to the observation that the therapeutic PSE treatment of birds infected by *S.* Enteritidis failed to eradicate *S.* Enteritidis from bird cecal tonsils relative to controls (**Table 5**). It may be that PSE treatment of extant infections rapidly selected for PSE resistance in *S.* Enteritidis bacteria or that *S.* Enteritidis that had previously colonized the treatment animals were resistant to infection (Higgins et al., 2008). This experiment highlights the importance of phage administration as a prophylactic prior to *S.* Enteritidis infection is a more effective strategy than phage administration as a treatment following *S.* Enteritidis infection (**Table 5**). Presumably, prophylactic PSE treatment can prevent establishment of *S.* Enteritidis infection, but is relatively ineffective against established *S.* Enteritidis infections.

In another experiment, phage PSE was applied to treated birds, either orally or via the vent lip, for 3 days prior to infection with *S.* Enteritidis. Following *S.* Enteritidis challenge, all birds were tested for *S.* Enteritidis infection periodically across 35 days. In birds treated with PSE orally, *S.* Enteritidis was detected in

the cecal tonsils 6 h following *S.* Enteritidis challenge, but not subsequently (**Table 6**). Some birds treated with phage PSE via the vent lip periodically tested positive for *S.* Enteritidis across the study period, but the majority remained free of *S.* Enteritidis (**Table 6**). Birds in the negative control (no treatments) and birds receiving only bacteriophage treatment remained free from contamination with *S.* Enteritidis across the entire study period (**Table 6**). These results imply that the bird quarantine was effective and no cross contamination occurred during the entire experimental period. Significantly, while both oral and vent lip treatment routes reduced *S.* Enteritidis infection compared to the positive control, the oral treatment route was more effective than the vent lip method in treatment of *S.* Enteritidis infections.

Although some studies suggested that it might be necessary to employ cocktails of bacteriophages to provide protection against *S.* Enteritidis (Atterbury et al., 2007; Borie et al., 2008), our study demonstrates that treatment with a single phage type can effectively prevent *S.* Enteritidis colonization. For practical and economic reasons, employing a single phage to achieve therapeutic effect is more desirable than cocktails that contain several types of phage (Sulakvelidze et al., 2001).

CONCLUSION

Phage PSE shows great promise for the prevention and treatment of *S.* Enteritidis infection, and it may be a plausible alternative to antibiotics for the reduction of *S.* Enteritidis shedding in poultry. Phage PSE was most effective when administered prophylactically prior to *S.* Enteritidis infection than as a treatment for established *S.* Enteritidis infections. Although we observed a reduction in *S.* Enteritidis infection in birds prophylactically treated with phage PSE via the vent route, our results indicate that administration of phage PSE via oral route is most effective.

HIGHLIGHTS

Phage PSE persistence was observed in the absence of *S.* Enteritidis challenge. Administration of phage PSE as a preventive agent could reduce the *S.* Enteritidis colonization more effectively than post challenge administration. Administration of phage PSE via oral route is most effective for reducing *S.* Enteritidis colonization.

AUTHOR CONTRIBUTIONS

MA and MK designed the study, performed experiments, analyzed the data and wrote the manuscript. SR and JD reviewed the manuscript. All authors read and approved the final manuscript.

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Bacteriophages against *Serratia* as Fish Spoilage Control Technology

*Igor Hernández**

AZTI-Tecnalia, Food Research Division, Parque Tecnológico de Bizkaia, Derio, Spain

Bacteria of the genus *Serratia*, mainly *S. proteamaculans* and *S. fonticola*, are important spoilage agents in Atlantic horse mackerel (*Trachurus trachurus*). In order to evaluate whether bacteriophages against *Serratia* could delay the spoilage process, 11 viral strains active against this genus were isolated from food and best candidate was applied to fresh mackerel filets. All the phages belong to the *Siphoviridae* and *Podoviridae* families and were active at multiplicity of infection (MOI) levels below 1:1 in Long & Hammer broth. The ability of phage AZT6 to control *Serratia* populations in real food was tested in Atlantic horse mackerel extract and applied to fresh mackerel filets. Treatment with high phage concentration (MOI 350:1, initial *Serratia* population 3.9 ± 0.3 Log cfu/g) can reduce the *Serratia* populations up to 90% during fish storage (a maximum of 6 days) at low temperatures (6°C). Bacterial inhibition was dependent on the bacteriophage dosage, and MOI of 10:1 or lower did not significantly affect the *Serratia* populations.

Keywords: phagotherapy, *Serratia*, fish spoilage, shelf life, *Trachurus trachurus*

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Pilar García,
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Lorena Rodriguez-Rubio,
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María Ángeles Tormo-Mas,
Researcher Ramon y Cajal, Spain

****Correspondence:***
Igor Hernández
ihernandez@azti.es

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INTRODUCTION

Bacterial viruses (bacteriophages) are considered to be the most abundant organisms on the planet. Their ability to reduce bacterial loads has led them to be suggested as therapies for human and animal microbial infections (Brüssow, 2012), but other applications are also possible. In food technology, phages have been proposed for food spoilage control (García et al., 2008; Mahony et al., 2011), and selected phages have been tested in food contaminated with *Salmonella* (Leverentz et al., 2004), *Listeria* (Leverentz et al., 2004; Carlton et al., 2005), or *Escherichia coli* (Carter et al., 2012). However, few references describe phages as a strategy against spoilage bacteria for shelf life extension of food. Bacteriophages against spoilage bacteria have the advantage that natural resistance of bacteria to lysis by phage is not a critical safety concern, while even modest reduction of specific microorganisms can significantly prolong food's shelf life. To the best of our knowledge, the first reference of phages as food "spoilage control agents" was from Ellis et al. (1973), who worked on milk storage. Later, other authors described phages that were active against *Pseudomonas* in beef (Greer and Dilts, 1990) and in milk (Sillankorva et al., 2008), *Listeria brevis* in beer (Deasy et al., 2011), *Brochothrix thermosphacta* in pork (Greer and Dilts, 2002), and *Shewanella* in fish (Li et al., 2014). In most cases, the target bacteria were inoculated in the food matrix, and the activity against naturally presenting bacteria was not considered; hence, complicates the transfer of the technology to the food industry.

Abbreviations: MOI, multiplicity of infection; Pfu/ml, plaque formation units per milliliter.

In previous work, Alfaro and Hernández (2013) determined that *Serratia* is a dominant bacterial genus (over 25% of the total bacterial load) at sensorial rejection time of Atlantic Horse Mackerel (*Trachurus trachurus*) filets stored in modified atmospheres (MAPs), and the increase of these bacteria during fish storage has been related to their fresh shelf life (Alfaro et al., 2013). Many bacterial species have been associated with formation of biogenic amines (De Filippis et al., 2013), and they are part of the spoilage microflora of diverse foods (Doulgeraki et al., 2012). The *Serratia* genus are Gram negative, facultative anaerobic bacteria, which grow in a broad range of temperatures and substrates, including plant surfaces, soil, water, and food products such as fruit juices and fish (Garrity et al., 2005). Many species have been related with food spoilage, and some of them (mainly *S. marcescens* and *S. liquefaciens*) have been described as opportunistic human pathogens (Mahlen, 2011).

Bacteriophages for *Serratia* have been purified before from sewage effluent, mainly using strains of *S. marcescens* as targets. Those virons have a relatively wide broad range of action, and are even able to infect bacteria of genera other than *Serratia* (Prinsloo and Coetzee, 1964; Prinsloo, 1966; Evans et al., 2010). Phages against *Serratia* have been used as molecular biology tools (Petty et al., 2006) and as models for viron abundance estimation in soil (Ashelford et al., 1999, 2003). To the best of our knowledge, no utilization in the food industry has been reported.

This paper describes the isolation, selection, and characterization of bacteriophages against *Serratia* species, and the evaluation of their ability to control growth of naturally occurring *Serratia* during spoilage of Mackerel filets. The research reported here provides an example of the potential and limitations of the application of bacteriophages to retard bacterial spoilage in foods.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

Serratia fonticola and *S. proteamaculans* strains were isolated in our facilities from spoiling Atlantic horse mackerel and were characterized by 16S RNA sequencing as previously described (Alfaro and Hernández, 2013). Two strains of *S. fonticola* (SFO001 and SFO002) and six strains of *S. proteamaculans* (SPR001, SPR002, SPR004, SPR005, SPR006, and SPR009) were used for phage isolation and characterization. Two *S. marcescens* strains (CECT 854 and CECT 977) were purchased from the Spanish type culture collection (CECT).

Long & Hammer agar and Long & Hammer broth (Koutsoumanis and Nychas, 1999) were used as general propagation media for *Serratia*. Strains were cultivated for 24 h at room temperature (22–25°C) in aerobic conditions. Bacterial populations in fish samples were quantified using: (i) *Serratia* selective agar (SSA) prepared as described by Starr et al. (1976) for enumeration of *Serratia*. Incubations were aerobic at 20°C. (ii) Triptone soya agar (TSA) incubated aerobically at 30°C for 24 h for total bacterial counts. (iii) Long & Hammer agar incubated aerobically at 12°C for 72 h for psychrotropic total bacterial counts. Peptone water was used for decimal dilutions.

Spoilage-related bacteria previously isolated and characterized from Atlantic horse mackerel (Alfaro and Hernández, 2013) were used to study the phage specificities. *Carnobacterium maltaromaticum*, *Shewanella putrefaciens*. *Vibrio* ssp., and *Yersinia intermedia* strains were used for phage specificity determinations. Bacteria in exponential growth phase (in TSB) were treated with phages of interest (MOI 1000:1), and growth was controlled by optical density at 600 nm ($OD_{600\ nm}$) and by colony count in TSA (incubated at 30°C for 24 h).

Phage Isolation and Enumeration

The *Serratia* bacteriophages were isolated from cheese whey obtained from artisanal cheese producers. Briefly, whey samples were mixed with an exponential (OD_{600} = 0.250–0.300) growth culture of SFO001 (whey:culture: 1:1) and incubated at 20°C for 7 h. These phage-rich samples were mixed with chloroform (10:1 v/v), centrifuged (3 min, 14.000 × *g*) and the water fractions were transferred to sterile tubes. Water fractions (or their decimal dilutions) were mixed (1:1) with high-density SFO001 culture (OD = 1.5) and with Long & Hammer soft agar. The mixture was smeared over a plate of Long & Hammer agar and incubated at 20°C for 24 h until plaques were visible. For further purifications, one plaque from each plate was extracted, dissolved in peptone water and treated as described above for whey samples. Three purification cycles were performed for each phage.

Phage enumeration was done with a spot test and double-layer agar technique (Kropinski et al., 2009; Mazzocco et al., 2009), using exponentially growing SFO001 as sensible strains. Plates (Long & Hammer agar and soft agar) were incubated at 25°C for 24 h. Samples of 10 µl of phage dilution were used for the spot tests. Phage dilutions were done in sterile saline–magnesium–gelatine buffer (SGM, 0.1 M NaCl, 1 mM $MgSO_4$*$7H_2O$, 50 mM Tris–HCl and 0.01% gelatine, pH = 7.5).

SPR009 has a high growth rate at a broad-range of growth temperatures, which facilities the use of this strain for phage-activity characterization in liquid medium. Long & Hammer broth was used.

For stock production of phages, they were cultivated with exponentially growing target strain (SPR009) in fresh Long & Hammer broth at 20°C for 24 h. Bacteria were hydrolyzed and removed by mixing the culture with chloroform (10:1), vortex and centrifuged (5 min, 25000 × *g*). Supernatant was spread on a Petri plate under sterile air for 15 min and stored at 4°C or at −80°C (20% glycerol added) until used. When high volumes of bacteriophage were necessary, samples were purified and concentrated as described by Sambrook and Russell (2001), using modified Long & Hammer broth (gelatin concentration was reduced to 1% and 10 mM $CaCl_2$ was added) as cultivation medium and PEG6000 as precipitation agent.

Phage Characterization

For molecular characterization, DNA was isolated according to the method described by Sambrook and Russell (2001). For restriction endonuclease analyses, DNA was digested with EcoRI (New England Biolabs) according to the manufacturer's recommendations. Fragment lengths were verified with gel electrophoresis (0.8% agarose in TAE buffer, 70v; Biorad, Madrid,

Spain) and visualized in a BioDoc-it imaging system (UVP, Upland, CA, USA). Generuler 1-kb DNA leader was used as a calibration standard (VWR, Madrid).

Phage morphology was studied by transmission electron microscopy (TEM). For purification, phage dilution (estimated as 10^{12} pfu/ml) was centrifuged (1500 × *g*, 60 min, 4°C), and the pellet was dissolved in sodium acetate solution. Centrifugation was repeated, and the pellet was finally dissolved in sodium acetate. For sample preparation, 5 μl of phage dilution was fixed in an Agar S-160 carbon rack temporally hydrophilized by glow discharge (20 s, 800v DV in vacuum conditions). Phages were stained with uranyl acetate 1% (twice, 30 s each). Phage morphology was examined with TEM (Philips CM120 biofilter) and photographed with an Olympus SIS "Morada" camera. Phage morphology and dimensions (capsid diameter, tail length, and width) were analyzed from electron micrographs with ImageJ software (Rasband, Ver 1.48).

Phage lytic spectra were determined using fresh phage propagated in SFO001 and double agar layer technique as described above. Plaques were counted after 24 h at 25°C.

Evaluation of Phage Activity in Fish Broth and Filets

For fish broth preparation, five fresh Atlantic horse mackerel were eviscerated, cut in small pieces and mixed. Then 1 kg of the fish was boiled for 10 min in 1.0 l of distilled water. The broth was filtered through 1 mm pores and autoclaved 15 min at 110°C. Fresh inoculum of strain SPR009 was used to inoculate the broth (final concentration (3.9 ± 0.2 log cfu/ml) and AZT6 phage was inoculated 10 min later at 6 or 20°C. After 5 days, a 1 ml sample was removed for bacterial and phage concentration determinations. Bacterial load was estimated on Long & Hammer agar (25°C, 24–48 h), and phage load was determined by the spot-test technique described above. Each experimental condition was studied in triplicate.

For antimicrobial activity on fish filets, *T. trachurus* filets were used as a model food matrix. These filets were not inoculated with bacteria, and the results reflect reduction of natural microbiota. Two fresh fish were supplied by each of two different local markets, eviscerated, heads-removed under sterile conditions and the bodies divided along the vertebral bones in two similar portions (in total eight "filets"). Four filets were submerged for 60 s in AZT6 phage solution (SMG buffer with different concentrations of phages), dried for 60 s under sterile air and stored in aseptic 140-mm Petri plates with plastic covers. Samples were stored aerobically at 6°C in a temperature-controlled chamber. After 3 or 6 days at 6°C, 5–10 g of fish from each filet were aseptically removed, homogenized in APT (fish:broth ratio was 1:10) using a Stomacher® blender (6 × {20″ on and 10″ off}) and used for bacterial counts.

Statistical Analysis

PSPP V0.6.2 (Free Software Foundation, Inc.) was used for analysis of variance (ANOVA) and least significant difference

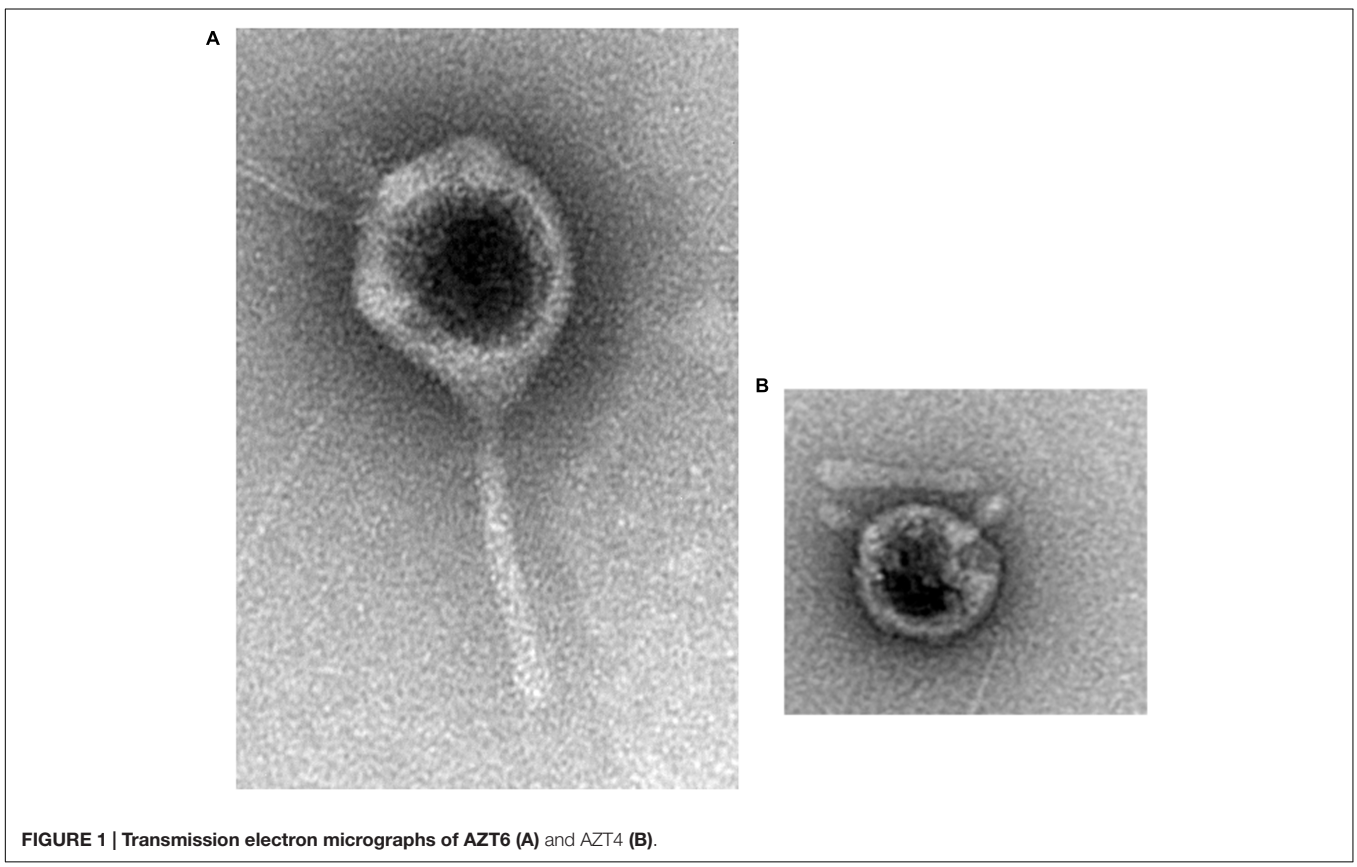

FIGURE 1 | Transmission electron micrographs of AZT6 (A) and AZT4 **(B)**.

(LSD) statistical procedures. A confidence interval of 95% ($p \leq 0.05$) was used.

RESULTS AND DISCUSSION

Phage Isolation and Characterization

Working with *Serratia fonticola* SFO001 the as target strain, 11 phages were isolated from cheese whey samples using the double-agar layer technique. Initial phage purification did not require Ca^{2+} addition, but addition of 0.5 mM Ca^{2+} increased the plaque size after the last purification cycle.

Isolated phages consistently produced clear plaques on Long & Hammer agar inoculated with *Serratia* SFO001 and, therefore, the whey phages were classified as virulent. Analysis showed that DNA of all of the isolated virons was double stranded and produced different DNA digestion profiles with EcoRI (results not shown). We discarded duplicate isolations. Estimated DNA size ranged from 50 to 70 Kbp.

Phages AZT1, AZT2, AZT3, AZT5, AZT6, and AZT9 have icosahedral heads (80–90 nm) and long non-contractile tails (100–120 nm). In contrast, AZT4, AZT7, AZT8, AZT10, and AZT11 presented spherical heads (75–90 nm) and extremely short tails (10–15 nm). All the isolated virons have lytic activity after 10 min of incubation in chloroform, suggesting that a lipid envelope is not a functional requirement. The first group would be classified as type B in Bradley's classification (Bradley, 1967), and they probably belong to the *Siphoviridae* family. The second group would be C-type phages (Bradley, 1967) classified with the *Podoviridae*. **Figure 1** presents the typical conformation of each type of phage. Other authors have demonstrated the highly variable morphology of the *Serratia* phages, even when they are isolated from the same source (Bradley, 1965; Ashelford et al., 2003).

Maximal phage titrations in the medium after a single cultivation round were between 6.9 and 9.3 Log pfu/ml (Supplementary Table 1). All bacteriophages were active after 30 days at −80°C with losses of viability less than 2.0 Log pfu/ml, except AZT5 and AZT8 that lost viability by more than five orders of magnitude during ultrafreezing. Similar activity losses in pure phage solutions were observed after 30 days at 4°C (Supplementary Table 2).

Bactericidal effects depend on MOI and, in most of the cases, effective MOI at 20°C ranged over five orders of magnitude (**Figure 2**, AZT3 as an example). An MOI close to 100:1 produced an initial decrease in bacterial concentration, followed by a normal growth curve. Lower phage doses (when activity is detected) permit initial bacteria growth, followed by decreases in bacterial concentration in later phases (**Figure 2**). In all cases, MOI of 1:1 or higher resulted in significant bacterial reduction (i.e., Supplementary Figure 1 for MOI 1:1) and doses with MOI smaller than 1:1000 were not tested. In most cases, no significant differences were observed in the phage activity while varying calcium concentration (up to 10 mM Ca^{2+}, Supplementary Figure 1) in broth. However, significant increases of inhibitory activity were observed using phages AZT7 and AZT9 when Ca^{2+} was increased over this concentration (results not shown).

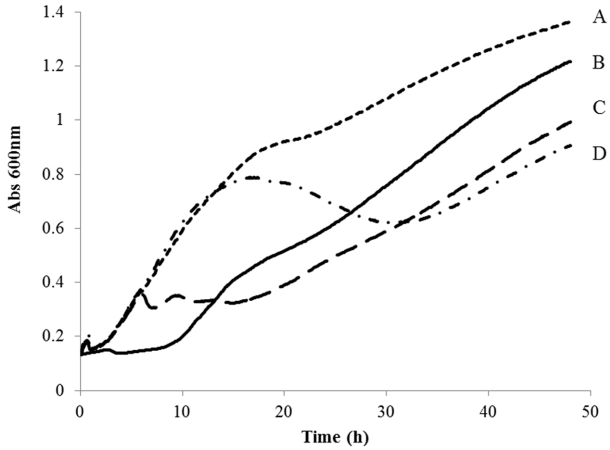

FIGURE 2 | Optical density (OD_{600}) evolution of strain SPR009 cultivated in Long & Hammer broth at 20°C treated with cultures AZT3 at different MOI. (A) No phages, **(B)** 100:1, **(C)** 1:1, **(D)** 1:1000.

Lytic spectrum (**Table 1**) showed high diversity among isolated phages. AZT6 showed the highest activity against four strains, while AZT5 is specific for SFO001 and AZT1 and AZT8 have a narrow activity range. None of the phages were active against all the considered strains.

Two phages were active against *S. marcescens* strains. AZT9 reduced populations of CECT977 by 35%, and AZT4 reduced those of CECT854 by 46% when the bacteria were grown in TSB supplemented with 2 mM Mg^{2+} and 2 mM Ca^{2+}. That lytic activity was low, so those phages have only weak impacts on the tested *S. marcescens* strains. None of the isolated phages were active against both strains.

Only AZT6 was selected for further studies. Reasons to discard other isolations include quick recovery of maximal growth rate after treatment (phages AZT1, AZT2, and AZT3), no activity at low MOI (AZT4 and AZT9), low stability at −80°C (AZT5 and AZT8) and maximal titrations below 8.5 Log pfu/ml (AZT7, AZT10, and AZT11).

AZT6 Characteristics

Four strains of *C. maltaromaticum, S. putrefaciens, Y. intermedia* and *Vibrio* spp., isolated in our lab from spoiled fishes (Alfaro and Hernández, 2013), were treated at high MOI to test AZT6 phage specificity. For all strains of these bacteria, our results (not shown) showed that phage-treated and -untreated samples had similar kinetic growth parameters (Lag phase duration, growth rate, maximal yield). As expected, AZT6 was specific for *Serratia* and did not affect other genera of spoilage bacteria. AZT6 has a broad host range and produced lytic calves with both *S. fonticola* (SFO001 and SFO002) and *three S. proteamaculans* (SPR001, SPR006, and SPR009; **Table 1**).

AZT6 reduced the growth rate and delayed the stationary phase of SPR009 when that strain was cultivated between 4 and 20°C (MOI 1:1, results not shown), demonstrating activity in the temperature range of food storage. At higher temperatures, bactericidal effects disappeared after the initial 24 h, when

TABLE 1 | Lytic spectra of isolated phages against considered bacterial strains.

		Considered phage[a]								
Bacterial species	**Strain**	**AZT1**	**AZT2**	**AZT3**	**AZT4**	**AZT5**	**AZT6**	**AZT7**	**AZT8**	**AZT9**
S. fonticola	SFO001	+++[b]	+++	+++	+++	+++	+++	+++	+++	+++
	SFO002	–	+	–	+	–	++	–	–	–
S. proteamaculans	SPR001	+	–	++	–	–	+++	+	–	++
	SPR002	–	–	–	–	–	–	–	–	–
	SPR004	–	+++	–	–	–	–	–	–	–
	SPR005	–	–	+	–	–	–	–	–	++
	SPR006	–	++	–	–	–	+++	–	++	–
	SPR009	–	–	–	–	–	+++	–	–	–
S. marcensens	CECT854	–	–	–	++	–	–	–	–	–
	CECT977	–	–	–	–	–	–	–	–	++

[a]*Phage total count was 2 × 10^6 pfu/ml using SFO001 as sensible strain.*
[b]*+++, >10^5 pfu/ml; ++ 10^3 to 10^5 pfu/ml; +, 10^1 to 10^3 pfu/ml; –, no visible calves.*

bacteria had reached to stationary phase, but 48 h or longer was necessary to verify bacterial growth reduction at low temperatures.

Cultivation broth had an important effect on phage activity. In clear medium (Long & Hammer) MOI of 1:500 or higher produced significant reduction in bacterial counts, but in fish broth, an MOI of 1:3 was required to reduce growth of the *Serratia* population. Along with other factors, fish extract presents high numbers of particles in suspension that would reduce the activity of the bacteriophages.

Phage titration of AZT6 was stable during storage at cold temperatures. In pure culture, phage concentration was reduced 10-fold in the first 30 days at 6°C (from 8.9 ± 0.0 to 7.9 ± 0.2 Log pfu/ml), after which constant titration lasted up to 6 months at 4°C (from 7.9 ± 0.2 to 7.7 ± 0.2 Log pfu/ml). Ultra-cold storage (−80°C, 30 days) in the presence of glycine (20%) resulted in a titration reduction of an order of magnitude.

Phage Activity in Liquid Medium

In order to select the best strategy for increasing the shelf life of fish products stored at 6°C, different MOI were tested in Long & Hammer broth. As observed in **Table 2**, MOI of 20:1 or higher have a high impact on bacterial load during initial storage (1–3 days), but then their effect decreased up to day 7, when no reduction was observed. Lower MOI did not initially reduce the bacterial growth or have a significant effect after three or more storage days. These results would agree with the expected mode of action of bacteriophages: at high concentration, bacteriophages cause initially a high bacterial decrees and days after treatment, phage-resistant bacteria growth rapidly. In the other hand, low phage dosage requires some time to propagate the infection and to reduce bacterial count significantly.

Phage activity under real fish-storage conditions (6°C) was tested in fish extract medium using SPR009 as the sensible strain. High phage:bacteria ratios significant reduced the bacterial concentration, up to 99.9% of inhibition after 5 days (**Table 3**). Use of a MOI lower than 1:1 had no effect on bacterial concentration at the tested storage times. Addition of Ca^{2+} (up to 5 mM) to the fish extract did not affect phage activity (results not shown), similar to the results obtained in Long & Hammer broth.

TABLE 2 | Bacterial counts reduction (control – AZT6 treated samples, strain SPR009; Log cfu/ml) in Long & Hammer medium stored at 6°C using different MOI.

Time (days)	**MOI**			
	20:1	**1:1**	**1:5**	**1:500**
0	0.8*	0.0	0.0	0.0
1	0.4*	0.1	0.0	0.0
2	0.8*	0.8*	0.3	−0.1
3	1.1*	1.4*	1.3*	0.6*
4	0.3	0.8*	0.8*	0.6*
7	0.1	0.4	0.6*	1.0*

**Significant differences between treated and control samples ($p < 0.05$) at different sampling times.*

TABLE 3 | Bacterial counts reduction (control – AZT6 treated samples, strain SPR009, Log cfu/ml) in fish extract stored at 6°C for 5 days using different initial AZT6 and bacterial concentrations.

Initial bacteria (Log/ml)	**Initial phage (Log/ml)**	**MOI (phag:bact)**	**Bacteria reduction* (Log cfu/ml)**	**Bacterial reduction* (%)**
3.9	4.4	3:1	1.6	97.5
4.6	5.5	8:1	2.1	99.4
3.9	5.4	30:1	1.0	90.0
4.6	6.5	80:1	1.7	97.8
3.6	6.0	250:1	2.0	99.0
3.6	7.0	2500:1	2.9	99.9

**Bacterial reduction using the untreated samples as controls. In all cases, differences are statistically significant.*

Phage Activity in Fish Filets

Determine the specific counts of *Serratia* in a wild environment represented an analytical problem in this research. Medium SSA

has been described as selective for *Serratia* (Starr et al., 1976) and does not allow the growth of other representative strains of major bacterial genera in spoiled *T. trachurus*: *Yersinia, Photobacterium, Vibrio, Shewanella,* and *Carnobacterium*. Only *Serratia* formed visible colonies in that medium (results not shown), which supports that counts in SSA mainly were *Serratia*. Nevertheless, we cannot fully discount that other bacteria might grow in this medium.

Phage AZT6 was effective when applied to raw fish filets, and it can reduce of the *Serratia* load after 6 days by more than 90%. When fresh fish filets were treated with high phage–concentration solutions (7.8 Log pfu/g, MOI 350:1) results showed a significant reduction of bacterial counts in SSA after 3 (−0.4 Log cfu/g) and six storage days (−1.1 Log cfu/g) in treated samples compared to control samples (**Table 4**, bach 1).

As expected, reduction in *Serratia* counts in treated filet samples compared with untreated ones were parallel to significant reductions ($p < 0.05$) of total bacterial counts after 3 and 6 days in TSA agar (−0.7 and −0.8 Log cfu/g respectively) and after 3 days (0.6 Log cfu/g) in Long & Hammer agar (**Table 4**, bach 1). In this medium, no differences were observed after 6 days, probably because bacterial counts had reached their maxima. Other bacteria genus, like *Yersinia, Photobacterium* and *Carnobacterium,* are counted in Long & Hammer agar and have been identified as important spoilage bacteria in *T. trachurus* (Alfaro and Hernández, 2013). These genera can grow vigorously in refrigeration (Alfaro et al., 2013) and, presumably, compensate the decrease in *Serratia* counts. A combination of bacteriophages active against different species would be a good strategy to reduce total bacteria and to increase the product's shelf life. To the best of our knowledge, no examples of bacteriophage mixture against different genera in food have been published, but the concept has been proved successfully in pharmacology (Chan and Abedon, 2012) and further research would validate this strategy as food preservation technique.

When fish filets from other batches were treated with two phage dosages (MOI 100:1 or 30:1), the one with the highest phage:bacteria ratio showed significantly lower *Serratia* concentration after 3 days (−1.5 Log cfu/g), but loads were comparable after six spoilage days (**Table 4**, bach 2). These dosage effect differences were not detected in TSA or Long & Hammer agar (**Table 4**, bach 2).

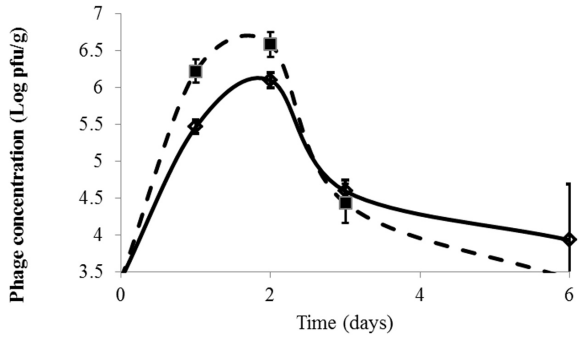

FIGURE 3 | Evolution of phage concentration (Log pfu/ml) in fish tissue during fish spoilage at 6°C. Fish 1 and Fish 2. Detection limit = 3.5 Log pfu/g. Phage concentatrion in not inoculated samples were always below 3.5 Log pfu/g. Initial value (time = 0) is <3.5 Log pfu/g. Visible initial point is 0.4 h after phage application.

Nevertheless, no count reductions similar to those in SSA, TSA, or Long & Hammer agar were observed in the filets treated with phages at MOI ≤ 10:1 (results not shown), even in the initial days. Phage AZT6 was active in broth at low MOI, and other researchers have described experience with phages active at low MOI (1:1) in pork adipose tissue (Greer and Dilts, 2002), but AZT6 required an MOI higher than 10:1, as have other phages described in the literature (Guenther and Loessner, 2011). It can be speculated that different strains of *Serratia* active on different fish samples could modify the bacterial response to phage treatments, that AZT6 is not able to multiply in or remain stable in the mackerel food matrix. As can be observed in **Figure 3**, when MOI ≤ 10:1 were used, AZT6 concentration in fish muscle increased in the first 2 days, and then decreased along fish spoilage; phage titrations went from the initial 5.9 Log pfu/ml to near the detection limit after 6 days. AZT6 titration in buffer solution was stable under the experimental conditions (time, temperature, pH), and no a clear cause was found to explain the decreases on the filets. Stability of other phages tested in food matrices were dependent on the food pH (Leverentz et al., 2001) or, as we expected for AZT6, they were stable in food matrices throughout storage (Guenther et al., 2009). Phages can be stabilized by absorbing them into water–base matrix (Murthy and Engelhardt, 2012) or microencapsulated (Ma et al., 2008;

TABLE 4 | Bacterial concentrations (Log cfu/g) in fish filets treated (MOI 350:1) or not with AZT6 after 3 and 6 days at 6°C.

		Day 3		Day 6	
Bach	**Medium**	**Treated**	**Not treated**	**Treated**	**Not treated**
1	SSA	5.3 ± 0.1*	5.7 ± 0.0	5.2 ± 0.1*	6.3 ± 0.3
	TSA	7.0 ± 0.0*	7.7 ± 0.2	7.3 ± 0.1*	8.1 ± 0.1
	L & H	7.4 ± 0.1*	8.0 ± 0.2	8.0 ± 0.1	8.1 ± 0.1
2	SSA	3.7 ± 0.2*	5.2 ± 0.1	6.3 ± 0.3	6.5 ± 0.3
	TSA	7.7 ± 0.4	7.0 ± 0.4	8.6 ± 0.4	8.7 ± 0.4
	L & H	8.1 ± 0.4	7.5 ± 0.4	9.1 ± 0.4	9.2 ± 0.4

**Significant differences between treated and untreated fish filets ($p < 0.05$) at each sampling times.*

Dini et al., 2012), that would result in a controlled bacteriophage release.

Phage adherence and penetration in fish samples are fast. Just 25 min after immersing fish filets in an AZT6 solution at 7.9 Log pfu/ml, we observed that phage concentration in the muscle increased from an initial value below 3.5–5.9 Log pfu/g (**Figure 3**). Phage AZT6 that settled on the filet surface penetrated by passive diffusion into the fish muscle that seems to be a fast and efficient process.

Our first attempt to test the phage activity in a solid matrix was to inoculate sterile fish cubes (autoclaved at 121°C for 15 min) with phage-sensitive SFO001 *Serratia* strain, and then treating them with high phage MOI (1000:1). After 24 and 96 h at 6°C, there was no bacteriophage effect on bacterial counts (results not shown). It is well-known that phage activity is favored in liquid media, and changes in muscle texture and reduction in food moisture would reduce phage activity. Phages are not motile organisms and their distributions are strongly dependent on conditions in food matrices. That is why most reported uses of bacteriophage involve adding them to liquid foods, like beer (Deasy et al., 2011) or milk (Modi et al., 2001; García et al., 2007). In only a few cases have they been shown to act on food surfaces (Guenther and Loessner, 2011).

Very few papers have been published about using phages for biocontrol of unintentionally contaminated foods. Probably, the report most similar to our study is that by Greer and Dilts (1990), who reported the inability of phages to control the spoilage of beef by wild *Pseudomonas*. The results presented in this paper not only demonstrate that the reduction of *Serratia* populations during spoilage is possible, but also demonstrated that many factors have to be adjusted for useful effective applications. Natural resistance is considered a problem for industrial applications. A mixture of phages providing broad spectra toward multiple target strains could to be an effective strategy. Another strategy would be that used by García et al. (2007), who transformed phages against *Staphylococcus aureus*, obtaining mutant phages that reduced otherwise bacteriophage-insensitive *S. aureus* variant levels up to 200-fold. A similar strategy would be useful for bacteriophage development against spoilage bacteria. Other factors, like application duration, have been reported as critical for effective phage activity in food (Leverentz et al., 2004).

We have described here a preliminary strategy for effective attenuation of *Serratia* growth during fish spoilage. Phage AZT6 is a promising candidate for application in order to reduce the *Serratia* population in freshly butchered fish tissues. It is active at low MOI, can be produced at high titration, and is stable for long periods under refrigeration. It shows bactericidal activity for multiple *Serratia* species, and we demonstrated its activity in real food matrices subject to spoilage. When MOI was 350:1, the bactericidal effect was up to 90% of the *Serratia* population in fish filets during storage at 6°C, linked with a bacterial count reduction in TSA and Long & Hammer agar. No Ca^{2+} additions were necessary for maximum activity, which would simplify their application in the large-scale fish-processing systems.

AUTHOR CONTRIBUTIONS

IH was responsible of the conception and design of the work, the acquisition, analysis and interpretation of the data presented in this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00449/full#supplementary-material

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REVIEW
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Phage Therapy: Combating Infections with Potential for Evolving from Merely a Treatment for Complications to Targeting Diseases

Andrzej Górski[1,2,3], Ryszard Międzybrodzki[1,2,3], Beata Weber-Dąbrowska[1,2], Wojciech Fortuna[1,2], Sławomir Letkiewicz[2,4], Paweł Rogóż[1,2], Ewa Jończyk-Matysiak[1], Krystyna Dąbrowska[1], Joanna Majewska[1] and Jan Borysowski[3]*

[1] Bacteriophage Laboratory, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [2] Phage Therapy Unit, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [3] Department of Clinical Immunology, Transplantation Institute, The Medical University of Warsaw, Warsaw, Poland, [4] Katowice School of Economics, Katowice, Poland

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Edited by:
Peter Mullany, University College London (UCL), UK

Reviewed by:
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***Correspondence:**
Andrzej Górski
agorski@ikp.pl

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Antimicrobial resistance is considered to be one of the greatest challenges of medicine and our civilization. Lack of progress in developing new anti-bacterial agents has greatly revived interest in using phage therapy to combat antibiotic-resistant infections. Although a number of clinical trials are underway and more are planned, the realistic perspective of registration of phage preparations and their entering the health market and significantly contributing to the current antimicrobial crisis is rather remote. Therefore, in addition to planning further clinical trials, our present approach of phage treatment carried out as experimental therapy (compassionate use) should be expanded to address the growing and urgent needs of increasing cohorts of patients for whom no alternative treatment is currently available. During the past 11 years of our phage therapy center's operation, we have obtained relevant clinical and laboratory data which not only confirm the safety of the therapy but also provide important information shedding more light on many aspects of the therapy, contributing to its optimization and allowing for construction of the most appropriate clinical trials. New data on phage biology and interactions with the immune system suggest that in the future phage therapy may evolve from dealing with complications to targeting diseases. However, further studies are necessary to confirm this promising trend.

Keywords: bacteriophage, phage therapy, antibiotic resistance, anti-phage antibodies, inflammation, reactive oxygen species, compassionate use

IS IT ETHICAL TO CONTINUE NOT PURSUING PHAGE THERAPY? (Henein, 2013)

On May 19, 2016 a review on antimicrobial resistance (AMR) commissioned by the UK Prime Minister was published. AMR – considered to be a challenge to health and our entire civilization – compared to terrorism and global warming – costs some 700,000 deaths annually and, if not controlled, would lead to 10 million deaths by 2050, exceeding the toll of cancer. To prevent medicine being cast back to the dark ages, the review suggests a number of actions promoting

alternatives to drugs. Interestingly, phage therapy has been placed at the top of a table presenting possible alternative products to tackle infections (O'Neill, 2016).

Earlier this year another milestone document, also a review delivered by 24 scientists from academia and industry and commissioned by the Wellcome Trust, aimed at identifying the prospective therapeutic replacements for antibiotics, considered a number of key factors in this issue: (1) feasibility of informative clinical trials, (2) magnitude of medical potential, (3) likelihood and consequences of resistance, (4) level of current research activity, (5) likely time to registration, and (6) activities enabling validation and progression. Again, among the top ten approaches which the group considered merited attention, phage therapy was included, with earliest anticipated registration as of 2022 (Czaplewski et al., 2016).

The recent discovery of a plasmid-borne colistin resistance gene heralds the emergence of truly pan-drug resistant bacteria (McGann et al., 2016) and suggests that the competition between the prospect of a pre-antibiotic era and registration of phages as available medicinal products may be won by bacteria, with all the dramatic consequences for our civilization emphasized by the report mentioned above. This strongly suggests that our model of phage therapy applied as experimental treatment should continue to be implemented by other medical centers, in line with the recent suggestions. As Gill and Young have pointed out: "...we can see no compelling reason why phage therapy cannot be made more widely available on compassionate use (CU) grounds to patients afflicted with serious bacterial infections which are refractory to standard treatments. A Hirszfeld Institute model for such treatment could be implemented. As in Poland, the availability of such a treatment would not only save lives but allow for the collection of clinical data under well-documented clinical conditions within the USA" (Gill and Young, 2011). In a more recent article in *Science,* the authors recommend that: "major hospitals should establish validated phage collections for CU applications, where, in many instances, the bacterial pathogen has been identified and could be tested for sensitivity to a library of phages" (Young and Gill, 2015). This assumption was also fully supported by other authors, who have emphasized that introducing phage therapy into Western clinical practice in a collaborative, CU fashion would not require further deviation from the current standard of care (Kutter et al., 2015). What is more, our approach does not constitute market placement and practically places production of phage preparations for our patients use outside of the scope of European Medicinal Product Directive 2001/83/EC which has recently been fully confirmed by legal analysis reported by Verbeken et al. (2014, 2016). "The debate is no longer about the pros and cons of phage therapy, but rather about how we can move forward for patients to benefit from this therapy. We are proposing to set up dedicated public structures, National Reference Centers (NRC) for bacteriophage therapy. These NRC will pilot these treatments and put in place production of hospital-based bacteriophage solutions, and application protocols that will ensure adequate product quality, patient safety and monitoring of treatment efficacy" (Debarbieux et al., 2016). We could not agree more, as we have done so when our phage therapy center was established in 2005, and have been continuing our activities that have made it possible to gain invaluable experience derived from patient care and careful monitoring clinical, laboratory and immunological indices (Górski et al., 2012; Międzybrodzki et al., 2012). The establishment of our center and earlier activities of our Institute were facilitated by a 100 years rich experience of former Soviet Union Countries (especially Georgia and Russian Federation) covered by in-depth excellent reviews (Chanishvili et al., 2009; Kutateladze and Adamia, 2010; Chanishvili, 2016).

CLINICAL TRIALS VS. OBSERVATIONAL STUDIES AND EXPERIMENTAL THERAPY: THEIR ROLE IN FURTHER ADVANCEMENT OF PHAGE THERAPY

One should also be aware that – in addition to randomized clinical trials (RCT) – observational studies are an important category of study designs, considered to be the next best investigational method which may even yield comparable results (Benson and Hartz, 2000; Concato et al., 2000). One should cite here FDA Commissioner who has pointed out: "Although randomized trials perform an essential role in the development of therapies, we should not neglect the crucial and complementary role that can be played by high-quality observational studies... However, truly effective use of this volume of observational data will require considerable methodological development, including whether an observational study can provide sufficient evidence to render a randomized trial unnecessary" (Califf and Ostroff, 2015). In this context it is noteworthy that the use of unapproved drugs outside of formal clinical trials in patients posing difficult clinical dilemmas has been accepted in a variety of countries including the US, Canada, Australia, many European countries such as the UK, Germany, Austria, Switzerland, France, Italy, as well as Japan and China. Such treatment is most often called CU or expanded access (EA), and its basic rules vary by country, most often encompassing chronic, seriously debilitating or life-threatening disease, lack of effective approved drugs for use in a given patient, approval by an ethics committee, and informed consent. CU may involve the use of a therapeutic product at any stage of its development, including preclinical and early phases of clinical trials (Bedell, 2010; Whitfield et al., 2010; Walker et al., 2014). Thus, CU of phage preparations may be considered an alternative to phage therapy performed using preparations that would have been formally approved following successful completion of clinical trials.

Recently, a number of review articles covering various issues related to phage therapy (including completed and ongoing clinical trials, and regulatory and ethical issues at national, EMA and FDA levels) have been published; therefore those aspects will not be addressed here. Suffice it to say that the first randomized double blind, placebo-controlled phase I/II clinical trial on patients with otitis externa reported symptom amelioration and a decrease of mean *Pseudomonas*

aeruginosa titers in those patients associated with significant phage replication *in vivo* (a 200-fold titer increase), as well as a lack of adverse effects. However, a recently completed phase II trial in children with acute *Escherichia coli* diarrhea did not show superiority over the current standard of care. The causes of this clinical trial's failure are obscure at the moment; increased levels of *Streptococcus* in those children raise fundamental questions on the causative agent(s); in addition, the relatively short duration of phage administration (4 days) might also have contributed to the unsatisfactory results (Sarker and Brussow, 2016). In 2017 the current Phagoburn clinical trial evaluating phage therapy in the treatment of burn wounds infected with *E. coli* and *P. aeruginosa* should be completed, while Technophage has received FDA clearance to begin a trial of a phage cocktail for the treatment of infected chronic ulcers occurring in diabetic foot infections. New data on the efficacy of phage therapy are therefore on the horizon; however, new clinical trials addressing important clinical dilemmas (e.g., urinary tract infections) are urgently needed (Chan et al., 2013; Ly-Chatain, 2014; Letkiewicz, 2015; Pirnay et al., 2015; Vandenheuvel et al., 2015; Young and Gill, 2015; Chanishvili, 2016; Debarbieux et al., 2016; Expert round table on acceptance and re-implementation of bacteriophage therapy, 2016; Verbeken et al., 2016).

Our phage therapy unit has been accepting patients since 2005, so during the past 11 years, we have gained rich practical experience resulting from direct patient care and monitoring their clinical, laboratory and immunological parameters. Those observations were summarized in our report published 4 years ago (Międzybrodzki et al., 2012). In the subsequent years, we have analyzed the data derived from more than additional 150 patients, which essentially confirmed our earlier report, both with regard to the therapy efficacy (good results achieved in approximately 40% of cases). Most importantly, we have re-confirmed the safety of the therapy and indicated a minimal number of cases in which it should have been discontinued due to side effects. The fact that immunocompetence in patients with depressed immunity has been evaluated as antibody responses to phage administered by the intravenous (iv) route as well as historical data indicating that patients were treated using that mode of phage administration with outstanding efficacy and safety support our data and suggest that iv phage therapy using purified preparations should be seriously considered (Chanishvili, 2016; Speck and Smithyman, 2016).

Although current protocols of phage therapy carried out at our institute have not supplied formal proof of phage therapy efficacy – according to the requirements of evidence-based medicine – they nevertheless provided valuable clinical and laboratory data suggesting the optimal pathways for clinical trials as well as novel and interesting data on phage interactions with the immune system. For example, already 10 years ago, we described successful eradication of MRSA intestinal carrier status with subsequent eradication of genitourinary tract infection with the same pathogen (Leszczyński et al., 2006); this was later confirmed by good results of urinary tract infections using oral administration of bacteriophage preparations (Międzybrodzki et al., 2012). Recently, Galtier et al. (2016) from the Pasteur Institute using a mouse model and oral phage administration showed 1000-fold titer reduction of uropathogenic *E. coli*. As pointed out (Brussow, 2016), this could lead to successful application of phage against intestinal and by extension urinary pathogens. Our data strongly suggest that at least some forms of urinary tract infections can be treated with oral phage preparations targeting causative pathogens.

Studies *in vitro* and in experimental animals suggest that the use of phage cocktails may be superior to monotherapy with a single phage targeting a given pathogen because of a generally wider target range. In addition, the likelihood of the development of phage resistance in bacteria is much less likely using cocktails than single phages (Chan et al., 2013; Vandenheuvel et al., 2015). However, some of our therapeutic phages are sufficiently polyvalent as to cover more than 60% of target strains. Moreover, as emphasized earlier, we have achieved an approximately 40% rate of good results including approximately 20% eradication rate using phage monotherapy although the phage resistance phenomenon was occurring in the course of treatment (Międzybrodzki et al., 2012). Furthermore, our preliminary observations do not suggest clear clinical superiority of cocktails vs. monovalent phage preparations This finding is in agreement with the data of Brown et al. (2016) who showed that the use of a phage cocktail was not superior to using a single phage. Of note, high antiphage activity of sera was observed in 43% of patients treated locally with cocktails, in contrast to only 17% of patients on phage monotherapy, which suggests that antibody responses to phage therapy may vary depending on whether patients receive monotherapy or a cocktail of phages. In fact, phages may induce different levels of immunization, and antibody responses to some phages contained in cocktails may be exceptionally high; this suggests that such phages present in cocktails may induce adjuvant-like effects (Łusiak-Szelachowska et al., 2016). One should also keep in mind that the registration of a multicomponent phage cocktail should be considerably more complex than a monovalent preparation, as experienced by Pherecydes which was requested to demonstrate stability of all components of their elaborate cocktail (Servick, 2016).

As pointed out, our experience suggests that despite bacteriophage-resistant strain proliferation, phage therapy may be successful, which may depend on virulence reduction in such bacterial strains (lower growth rate, underexpression of virulence genes, loss of pathogen's ability to attach to human cells, markedly reduced lifespan (Leon and Bastias, 2015). Without the pressure of phages, resistant strains may revert to the parental phenotype or may be displaced by non-resistant virulent strains which may explain why prolonged phage therapy may be sometimes more efficacious than a shorter protocol. Our clinical data are confirmed by others (Capparelli et al., 2010) who demonstrated in mice that phage-resistant bacteria may not only be avirulent, but are also rapidly cleared by the immune system and, importantly, induce a balanced anti-inflammatory response (repression of transcription of the TNF-α and IFN-γ genes and induction of expression of the IL-4 and IL-6 genes). What is more, acquired phage resistance may be associated with

greater sensitivity to antibiotics. Those important data strongly suggest that – from the clinical point of view – the development of phage resistance by relevant pathogens should not always be considered as an undesired phenomenon, as it may cause offending bacteria to become increasingly antibiotic-sensitive and allow for renewed use of historically effective antibiotics that have been rendered useless by the evolution of antibiotic resistance. This approach has the potential to extend the effective lifetime of antibiotics in our drug arsenal and broaden the spectrum of those drugs, greatly reducing the burden on drugs of the last resort, preserving them for future use (Chan et al., 2013). The issue of combined use of phages and antibiotics is of evident clinical significance. Some experimental data *in vitro* and in experimental animals may suggest that such treatment could be superior to phages or antibiotics alone; this problem has recently been discussed in some detail (Torres-Barcelo and Hochberg, 2016) and therefore will not be elaborated here. Also, one can find some data in patients suggesting higher efficacy of such combined therapy (Kutateladze and Adamia, 2010; Chanishvili, 2016). Most proponents of this treatment highlight its potential. However, as rightly pointed out (Torres-Barcelo and Hochberg, 2016), there are also potential drawbacks of phage-antibiotic combinations such as the development of double-resistant variants, similar to the effects of antibiotic cocktails, which could have catastrophic consequences not only for patients thus treated but for further prospects of successful combat of AMR. Our policy has been to add antibiotics to phage treatment solely in polyinfections in which no phage was available to match an additional pathogen. Evidently, planned clinical trials involving phage therapy should also include combination treatment with antibiotics to provide more reliable data on this important clinical dilemma.

ANTI-INFLAMMATORY EFFECTS OF PHAGES

One of the most promising aspects of phage therapy is its remarkable anti-inflammatory action. We have noted a significant decrease in mean C reactive protein (CRP) values and leukocyte counts, with a similar tendency of erythrocyte sedimentation rate (Międzybrodzki et al., 2009). In some patients the reaction of CRP was dramatic and decreased from 50 to 5 mg/l within 2–3 weeks of the treatment even though complete eradication of infection was not achieved. This suggests that phage can exert its anti-inflammatory action by at least two mechanisms: one dependent on its well-known anti-bacterial action, and another which acts directly on phenomena responsible for the development of inflammatory processes. In fact, we have demonstrated that phage can diminish cellular infiltration of allogeneic skin transplants in mice and activation of the nuclear transcription factor NF-kappa B (which leads to expression of proinflammatory cytokines, chemokines, and adhesion molecules (Górski et al., 2006a). A short tail fiber protein, tail adhesion gp12, mediates adsorption of T4 phage to *E. coli*, binding LPS. Recently, our group demonstrated that recombinant gp12 counteracts proinflammatory effects of LPS *in vivo*, causing the reduction of serum IL-1 and IL-6 levels as well as a decrease of inflammatory infiltration in spleen and liver (Miernikiewicz et al., 2016). What is more, we have observed that phages and their surface proteins do not stimulate inflammatory mediator and reactive oxygen species (ROS) production when administered to mice (Miernikiewicz et al., 2013). This confirms and extends our earlier reports indicating that T4 phage lysates and its purified preparations induce only minimal levels of respiratory burst in whole blood monocytes and neutrophils while staphylococcal phage preparations do not stimulate the production of ROS at all (Borysowski et al., 2010). Furthermore, phages diminish ROS production induced by bacteria and endotoxin (Międzybrodzki et al., 2008), which highlights their potential in the treatment of sepsis (Weber-Dąbrowska et al., 2003). As shown by our group, phages do not induce granulocyte degranulation (Borysowski et al., submitted for publication), their administration to patients is not associated with leukocytosis (conversely, as stated earlier, they may reduce the number of circulating leukocytes in patients with bacterial infection; it is also noteworthy that phage therapy does not cause eosinophilia) (Międzybrodzki et al., 2012). In conclusion, our studies performed *in vitro* as well as *in vivo* in experimental animals and patients strongly suggest that phages may exert anti-inflammatory effects that can be useful clinically. The success of phage therapy depends on the ability of phages to migrate to infected tissues and achieve concentrations necessary to eradicate infection and exert anti-inflammatory action. In this regard, we proposed to engineer phages armed with tissue-specific peptides – this methodology, not necessarily involving genetic manipulations, may significantly enhance the effectiveness of phage therapy (Górski et al., 2015).

PHAGES AND THE IMMUNE SYSTEM

Górski and Weber-Dąbrowska (2005) proposed that phages may mediate immunomodulatory, probiotic-like functions and this phenomenon could be relevant in regulating local immunity in the intestinal tract where phages are an abundant part of the microbiome. What is more, phage translocation from the intestines might contribute to phages mediating such probiotic-like functions also in other parts of the body (Górski et al., 2006b). As the evidence on phage engagement in regulating immunity is accumulating, those data add credence to our hypothesis. In fact, our comprehensive review addressing the issue of phage interactions with the immune system and their possible practical implications, especially in relation to the therapy, highlights the potential role of phages as clinically useful immunomodulators. Our data indicate that about half the patients prior to phage therapy are immunodeficient; although the therapy may cause some fluctuations of immune parameters, its beneficial effects are not correlated with upgraded immunity (except for an increase of phagocytosis noted in some patients which also appears to have some positive prognostic value). Therefore, the therapeutic effects of phage therapy are

associated with its anti-bacterial and anti-inflammatory action rather than resulting from correction of depressed immunity, so the potential vaccine-like effect of phages is not responsible for their curative activity (Górski et al., 2012). Our data on monitoring phagocytosis by neutrophils and monocytes of patients on phage therapy suggest that the therapy may instead correct existing deficiencies in phagocyte functions (see above). In fact, we have shown that phages – both *in vitro* and *in vivo* – do not adversely affect the ability of phagocytes to kill bacteria – both standard strains as well as specific pathogens isolated from patients (Jończyk-Matysiak et al., 2015). Also, phages do not impair migratory activity of human phagocytes *in vitro* (Kurzępa, 2011), whereas they may markedly diminish tissue infiltration with those cells at the foci of inflammation (Górski et al., 2006a; Miernikiewicz et al., 2016).

An important clinical dilemma is whether phage therapy may be safe and efficient in immunosuppressed host (Borysowski and Górski, 2008). Our data obtained in patients with antibiotic-resistant infections who frequently have associated immunodeficiency confirm the value of the therapy in this syndrome. Also, phages have been successfully applied in cancer patients and renal allograft recipients (Borysowski and Górski, 2008). Those clinical observations were also confirmed experimentally by our group (Zimecki et al., 2010), who described the protective effects of phage therapy in immunodeficient mice subjected to myeloablative and immunosuppressive conditioning followed by bone marrow transplant and infected by sublethal and lethal dose of *Staphylococcus aureus*. Of note, we have shown that phage preparations do not enhance inflammatory processes in experimentally induced autoimmune disease in mice and may even have protective and therapeutic action (Międzybrodzki et al., unpublished data). These findings may suggest that phage therapy is also safe in patients with autoimmune disorders. If confirmed, those data should be especially relevant as such patients are especially prone to multidrug resistant infections.

ANTIBODY RESPONSES TO PHAGE THERAPY

Animal models allow for comprehensive studies of immune responses to bacteriophages *in vivo*. Induction of specific anti-phage antibodies has probably been the most extensively studied in mice, resulting in a multi-faceted description of this phenomenon. This comprises dose, schedule and route of administration effects, with regard to primary classes of immunoglobulins, and with reference to various immunogenicity of particular structural elements of bacteriophages.

A model bacteriophage T4 has been demonstrated to be able to induce specific antibody both after phage injection into peritoneum (Dąbrowska et al., 2014) and in long-term *per os* treatment (Majewska et al., 2015). In the experimental model of *per os* treatment, efficient induction of specific antibody production required long exposure of animals to the phage. The authors observed a significant increase in serum IgG on day 36. Once the IgG level reached a peak, it remained high throughout the experiment, even after the phage was removed from the diet. It did not, however, impact gastrointestinal phage transit. Interestingly, no clear IgM peak preceded the IgG boost, which is in contrast to immunization by parenteral applications, where a significant increase of IgM has been reported (Dąbrowska et al., 2014; Hodyra-Stefaniak et al., 2015).

A characteristic feature of oral administration is the secretory IgA production in the gut. In fact, this can also be observed as a result of bacteriophage administration *per os*, but again its induction requires long and high dose exposure to bacteriophage. An increase in secretory IgA was reported on day 79 of the oral treatment with T4. Importantly, the increase of phage-specific IgA in the gut correlated with the lack of viable phage particles detected in the feces. Thus, specific anti-phage IgA can be considered as a factor limiting phage viability in the gut (Majewska et al., 2015).

In order to draw a general conclusion about T4 phage immunogenicity for the needs of therapeutic approaches, an estimated adequate dose in humans was calculated: 2×10^{10} pfu per mouse corresponds to 7×10^{13} pfu per human patient daily (using a simplification of volumes as proportional to weight across species). The dosage used in therapeutic approaches in humans is usually much lower: in the 20th century, tablet or liquid formulations that were used in oral treatment of humans contained 10^5 to 10^{11} pfu/dose (Sulakvelidze et al., 2001), while daily therapeutic phage doses used in the Phage Therapy Unit of the Institute of Immunology and Experimental Therapy (IIET) in the years 2008–2010 ranged between 3×10^7 and 6×10^{10}pfu per patient (Międzybrodzki et al., 2012). Also in T4 phage safety tests conducted by Bruttin and Brüssow (2005) the total amount of phage preparations administered to human volunteers was much lower and equaled 9×10^7 pfu; no specific antibodies were detected following the safety tests. Taking into consideration the unusually high dosage and the time of continuous treatment that was necessary to elicit a humoral response (2 weeks in the case of IgG and as long as 2 months in the case of IgA), T4 phage immunogenicity in oral administration was defined as weak.

Microbiological assessment of bacterial fecal flora during the prolonged feeding of mice with T4 showed no substantial differences between phage-treated and control mice. The emergence of phage-resistant *E. coli* strains was observed in phage-treated mice very late: on day 92 (Majewska et al., 2015). This fact is important in the light of recent studies of the human microbiome that have demonstrated links between dysbiosis in the gut and numerous health problems, both located within the gastrointestinal tract and in other parts of the body (Kau et al., 2011). Animal studies suggests that the impact of orally applied phage on gut natural microflora is minimal.

Structural proteins of bacteriophages may differ in their individual immunogenicity. Furthermore, the route of administration may play a role in the resulting ability of

the proteins to induce specific antibodies. T4 phage proteins reported as highly immunogenic when applied intraperitoneally were major capsid protein gp23 and highly antigenic outer capsid protein gpHoc (Dąbrowska et al., 2014). However, oral administration of this phage resulted in immunization mostly to gpHoc and gp12 (tail spike) (Majewska et al., 2015). These data highlight the fact that route of administration plays a role in determining the fate of phage particles in the context of the specific humoral response. Gp12 is crucial in the process of phage adsorption and infection of bacterial host cells; therefore, the humoral response directed toward this particular protein may impair antibacterial properties of the phage and consequently impact the efficacy of phages as therapeutic agents. Hence, further studies aiming to identify the molecular basis for this response may, in the future, facilitate the optimal choice and design of phage use in therapy. On the other hand, a high-level humoral response may turn out to be beneficial in some scenarios. T4 phage has been effectively used as a phage display platform for foreign antigens, often displayed as Hoc fusion proteins (Rao group: Jiang et al., 1997; Sathaliyawala et al., 2006; Shivachandra et al., 2006, 2007). In this case, the ability of Hoc protein to induce a high-level humoral response may result in more efficient immunization to the fusion protein, which is highly desirable in vaccine development.

Since bacteriophages can be neutralized by specific antibodies *in vivo* and *in vitro*, a few possible mechanisms of phage inactivation have been considered. The most straightforward one was direct interaction of antibodies with phage proteins that are necessary for infection of bacterial cells; phage cannot attack bacteria since these proteins have been occluded by antibodies (Jerne and Avegno, 1956). However, anti-head immunization has also been demonstrated to reduce phage activity. In this case aggregation of phage particles as well as the antibody-dependent complement pathway were proposed as the mechanisms of phage inactivation (Dąbrowska et al., 2014).

Our recent papers have analyzed the practical issues of neutralizing antibody responses to phages in their clinical context based on the largest patient material ever available (Łusiak-Szelachowska et al., 2014, 2016). As noted, several key factors are responsible for those antibody responses: patients' immune status, route of phage administration, antigenicity of a given phage, and monotherapy vs. phage cocktails. Furthermore, the clinical significance of the production of anti-phage antibodies is unclear at the moment, as we have not confirmed a correlation between their appearance and therapy outcome. Conversely, good clinical results may be achieved with concurrent high serum neutralizing antibody levels against administered phage. Interestingly, anti-staph phage that effectively controls *S. aureus* growth and reduces bacterial viability both *in vitro* and in a skin infection mouse model loses its killing effect when the phage is cultured in the presence of human blood (Pincus et al., 2015). It may well be that local antibody interactions with phage at the foci of infection are more relevant for therapy success than serum antibody levels, whose raised values may – paradoxically – at least in some patients indicate a good clinical outcome signaling the recovery of the immune system and its more active participation in clearing infection. Our data suggesting that recovery of phagocytosis may be a good prognostic sign for therapy outcome provide food for thought for such hypothesis.

FUTURE PROSPECTS OF PHAGE THERAPY

It has been highlighted that phages – unlike classical antibiotics – are biological entities of incredible diversity and adaptability and many surprises may be in store (Young and Gill, 2015). The above mentioned data suggest that phages could interfere with some viral and fungal infections which could extend its potential therapeutic value beyond well-known antibacterial action. One could list here our recent data showing that T4 phage can inhibit infection of the target cells by an adenovirus (Przybylski et al., 2015) as well as the data from Stanford indicating that *P. aeruginosa* phage inhibits *Aspergillus* (Penner et al., 2016), which suggest that future applications of phage therapy may extend beyond its well known antibacterial action. In recent years, we have been facing unprecedented growth of interest in the human microbiome which has emerged as an important factor in human physiology and disease including obesity and diabetes, cancer and susceptibility to its chemotherapy, cognition and depression, etc. Therefore, manipulation of the microbiome is currently believed to have great potential for efficient therapy of disorders posing a challenge to medicine and civilization (Blaser, 2014). Phage-mediated immunomodulation of the enteric immune system and microbiome may be an important key to the success of this strategy (Górski et al., 2003, 2006b). One cannot thus exclude that the future phage therapy may be focused on our microbiome. Today, we can use phage therapy to combat urinary tract infections secondary to kidney stones which treats complications rather than a disease itself. Gut microbiota is unique in kidney stone disease (Kelsey, 2016). One could therefore envisage using phages to manipulate gut microbiome to treat and prevent the development of renal stones not only their complications. Moreover, phage ability to reduce the production of ROS and inflammatory processes (e.g., tissue infiltration by leukocytes) could be helpful to ameliorate the clinical course of patients with disorders where those phenomena are relevant (e.g., graft rejection, inflammatory bowel disease, etc.).

While the Canadian doctor F. d'Herelle discovered phages, another Canadian medical authority has emphasized: "The good physician treats the disease, the great physician treats the patient who has the disease" (Sir William Osler). A good example of this philosophy is the recent data suggesting that intensive glucose lowering protocols cause more harm than benefit in patients with type 2 diabetes (The Action to Control Cardiovascular Risk in Diabetes Study Group, 2008). Patients with antibiotic-resistant infections have complex clinical problems and frequently pose difficult clinical

dilemmas, resistant infection being an important, but not the sole component of their morbidity. The main purpose of a physician should be to provide optimal therapy considering all aspects of a patient's disorder, that is to heal the patient, not just eradicate infection (certainly not at any cost). Some reviews on phage therapy appear to disregard this principle; in this regard, one could cite an important recent opinion of Nature Medicine: "researchers need to reach back to the patients, but in a way that steers clear of giving medical advice" (Knoepfler, 2016). Phage therapy has the potential to go beyond from merely treating an infection in a patient to treating its causes and prevent further complications, a promise that requires further studies and confirmation. Tailoring medical treatment to the individual characteristics, needs and preferences of each patient fulfills the promise of a new era of medical product development referred to as personalized (precision) medicine (Food and Drug Administration, 2013). Phage therapy constitutes an excellent example of this novel trend.

AUTHOR CONTRIBUTIONS

AG drafted the main part of the manuscript, KD, JM, JB, EJ-M contributed parts of the manuscript, all authors revised the manuscript.

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OPINION
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The Developing World Urgently Needs Phages to Combat Pathogenic Bacteria

Tobi E. Nagel[1], Benjamin K. Chan[2], Daniel De Vos[3,4], Ayman El-Shibiny[5], Erastus K. Kang'ethe[6], Angela Makumi[7,8] and Jean-Paul Pirnay[3]*

[1] Phages for Global Health, Oakland, CA, USA, [2] Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, USA, [3] Laboratory for Molecular and Cellular Technology, Burn Wound Center, Queen Astrid Military Hospital, Brussels, Belgium, [4] Faculty of Medicine, Catholic University of Bukavu, Bukavu, Democratic Republic of Congo, [5] Biomedical Sciences, University of Science and Technology, Zewail City of Science and Technology, Giza, Egypt, [6] Department of Public Health Pharmacology and Toxicology, University of Nairobi, Nairobi, Kenya, [7] Department of Microbial and Molecular Systems, KU Leuven, Leuven, Belgium, [8] Center for Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya

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With the growing global antimicrobial resistance crisis, there is a critical need for alternatives to conventional antibiotics, especially in developing countries. Virulent bacteriophages (phages) represent a viable antibacterial technology that could be particularly beneficial, since phages are active against antimicrobial-resistant bacteria, easy to isolate from contaminated environments, and relatively inexpensive to produce. We discuss here examples of infectious diseases that significantly affect developing countries, phage applications that could be especially impactful in those settings, and special considerations for implementing phages in the developing world.

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****Correspondence:***
Tobi E. Nagel
tobi@phagesforglobalhealth.org

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DEVELOPING COUNTRIES ARE DISPROPORTIONATELY IMPACTED BY INFECTIOUS DISEASES

Bacterial infections cause more loss of life and health in developing nations than in wealthy ones. As an example, infection with the bacteria *Campylobacter* is associated with an average case fatality rate of ∼0.1% in developed countries, but in Kenya reportedly 8.8% of infected individuals die, with most of those deaths occurring in children (O'Reilly et al., 2012; The Global View of Campylobacteriosis, 2013). A 2014 report commissioned by the UK Prime Minister predicts that by the year 2050, almost 10 million people will die from antibiotic-resistant infections annually: roughly 4.73 million in Asia and 4.15 million in Africa, in contrast to 0.39 and 0.32 million in Europe and the US, respectively (Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, 2014). These numbers are actually underestimates, since they are based on data available for only three of the seven bacteria that the World Health Organization has deemed concerns.

Developing nations are also less able to prevent infections. Countries categorized as lower middle income countries (LMIC)—defined as those with less than $4125 gross national income *per capita*—are more likely to lack clean water and have sanitation and hygiene problems. Not only are greater numbers of people affected by bacterial pathogens in LMIC, infected individuals are often more vulnerable. Malnourished, immunocompromised, and HIV-positive patients have more severe illnesses and greater risks. Given the public health and antibiotic resistance problems in the developing world, alternative treatment modalities are urgently needed. Here, we describe how phages are particularly appropriate for LMIC.

Abbreviations: LMIC, lower middle income countries.

PHAGES ARE WELL-SUITED FOR APPLICATIONS IN DEVELOPING COUNTRIES

For over 100 years, virulent phages have been used in the former Soviet Union as alternatives to and alongside traditional antibiotics (Abedon et al., 2011). Therapeutic phages are naturally occurring viruses that can be selected to kill only specific bacterial species or strains while leaving other, helpful bacteria and mammalian cells unharmed. Thus, unlike broad-spectrum antibiotics, they spare the commensal microbiota (e.g., in the gut)—a characteristic particularly important for malnourished and immunocompromised individuals. In addition, they can be effective against antibiotic-resistant bacteria. Phages can be easily isolated from environments enriched in targeted bacteria, such as hospital waste or sewage water, with tools readily available to people in LMIC. In principle, phage products can also be developed faster and more cost-effectively than conventional drugs, and they can be dry powder formulated so that they require no refrigeration (Semler et al., 2012).

CURRENT AND POTENTIAL USES OF PHAGES

Phage applications span a broad range, from food systems to animal husbandry and clinical therapies. Generally, only lytic phages are considered suitable for biocontrol or therapeutic applications. Unlike temperate phages, lytic phages obligately lyse bacteria and do not mediate the exchange of virulence or antibiotic resistant determinants between bacteria.

Food Decontamination

Methodologies that address foodborne diseases are particularly important for LMIC: many developing countries lack reliable refrigeration and good hygiene practices, providing ample opportunities for bacteria to grow. In Western countries, several phage products are currently approved for the control of food pathogens, including *Listeria monocytogenes, Salmonella,* and *Escherichia coli* (Naanwaab et al., 2014). They are used as sprays to decontaminate fruits, vegetables, cheese, fish, poultry, and meats. If such phage products were available in developing countries, their benefits could be even greater. For example, given the scarcity of dependable refrigeration in many LMIC, meat products are ideally sold within 24 h of slaughter. Thus, any antibacterial product that could increase the shelf life by even 1 day could have significant impacts not only on public health, but also on profitability for meat sellers.

Veterinary Applications

Phages could also be employed to decrease bacterial loads in animals before they reach slaughterhouses. For instance, *Campylobacter* is resident in the intestinal tracts of up to ∼75% of poultry in many countries (Coker et al., 2002). While it causes little harm to chickens, it is the leading bacterial cause of gastroenteritis in humans worldwide. Data from Egypt shows that in households with backyard poultry that are contaminated with *Campylobacter,* children are almost four-fold more likely to be infected with the bacteria (El-Tras et al., 2015). Numerous studies have demonstrated that adding *Campylobacter* phages to chicken feed can decrease bacterial levels in experimental bird models by several orders of magnitude, presenting an attractive biocontrol strategy (Connerton et al., 2011). However, any applied phages would need to be active against the range of bacterial strains present in poultry flocks, and the environmental impacts of large-scale phage application should be studied in greater depth (Marotta et al., 2015). If not managed properly, bacteria could develop broader resistance to phages. Multiple approaches can be employed to minimize such resistance, including adapting the phages *in vitro*, utilizing phage cocktails, and switching to different phages at regular intervals (Chan et al., 2013). Phage preparations, especially for biocontrol applications, will likely need to be tailor-made for specific settings and will require periodic updates to address pathogen diversity as well as evolutionary changes in bacterial populations (Hagens and Loessner, 2010). Ideally, banks containing different, well-characterized phages should be set up and regularly updated with new phages.

Major economic markets in developing countries could also benefit from phages. A prime example is the dairy industry, in which bovine mastitis (inflammation of the mammary glands) is the leading cattle disease worldwide (Dias et al., 2013). In India, the largest producer of milk globally, bovine mastitis decreases annual milk yields by up to 70%, with financial losses of over $1 billion each year (Sharma et al., 2012; Padhy et al., 2015). Since bovine mastitis is mainly caused by bacteria (*Staphylococcus* and *Streptococcus*) where antimicrobial resistance is increasing, alternatives to antibiotic treatment are urgently needed. Bovine infections also affect human health: low levels of contamination can be difficult to detect, often leaving bacteria to be propagated and transferred to humans through milk products (Hameed et al., 2006; Wareth et al., 2014). By decreasing such infections in dairy cattle, phages could potentially contribute to both the financial and medical health of populations in LMIC.

Human Treatment

Over the past century, phages have been administered in some regions directly to people, either as prophylaxis or to treat active infections (Abedon et al., 2011). In the 1960s, phages were tested for the prevention of seasonal *Shigella* infections in the country of Georgia. More than 30,000 children received *Shigella* phages or a placebo, and there was a statistically significant reduction in the *Shigella* infection rate in the phage-treated group (Kutter et al., 2010). Estimates indicate that *Shigella* species infect 164.7 million people annually—with 163.2 million of those cases occurring in developing countries, resulting in 1.1 million deaths each year (Kotloff et al., 1999). Since *Shigella* strains now exhibit worrying rates of resistance to antibiotics, even to third generation cephalosporins, there is significant motivation to explore using *Shigella* phages in LMIC.

In the 1920s and 1930s, several studies were conducted in India testing the efficacy of orally delivered cholera phages

(Summers, 1993). In one study series involving more than 800 patients, there were consistently fewer deaths in the patients who received phages. Another study tested the ability of phages to control cholera outbreaks in hundreds of villages. Two comparable (in terms of yearly cholera outbreak patterns) yet geographically separate village regions were monitored, with one of the regions receiving phage treatment and the other not. In the treated region there were no epidemics during the 6-year course of the study, whereas in the untreated region there were seasonal epidemics in the first 3 years. After that, the government ordered phage treatment in both regions, with almost no cholera cases reported during the following 3 years. The discovery and early promise of antibiotics in the 1940s eventually led to the abandonment of phage therapies such as these. Now, given the problems of antimicrobial resistance, it is appropriate to give phages another look.

While historical phage studies were not always conducted according to the rigors of modern clinical trials, they do strongly suggest that phages could be useful in outbreak settings, such as in the Haiti cholera epidemic of 2010, which affected more than 700,000 people. In such situations, phages could help fill gaps that are not fully addressed by current vaccine and hygiene strategies. For example, phage treatment could be particularly helpful for non-vaccinated populations, since phages could work in parallel with vaccines, beginning to kill intestinal bacteria within hours, while vaccines take weeks to provide full immunological protection. Phages should also be beneficial to immunocompromised individuals, whose immune systems often are not fully responsive to vaccines. And while vaccines require refrigeration, lyophilized phages can be stored at room temperatures (Semler et al., 2012). Thus, phages could be an effective addition to the arsenal of tools already available to combat cholera outbreaks.

REGULATORY CONSIDERATIONS

To fully realize the public health benefits of phages in LMIC, each country will need to establish appropriate regulatory guidelines. Unfortunately, traditional regulatory systems are not well-suited for the development of sustainable phage products. For example, most regulatory structures focus on drugs that are composed of a single chemical entity, whereas phage preparations would ideally be custom-made and updated at regular intervals to both enhance their efficacy and minimize the development of bacterial resistance. Since phages are naturally occurring entities, many experts have argued that a different type of regulatory process should be established for phages, perhaps something closer to that governing probiotics. One alternative approach currently being explored at the Queen Astrid Military Hospital in Belgium is to produce phages in compliance with a formal monograph describing the standards by which the phage quality will be judged for specific applications (e.g., pharmaceuticals, dietary supplements, or food ingredients; Merabishvili et al., 2009).

IMPORTANCE OF LOCAL CULTURAL CONTEXTS

Even though phages have a long history of being used as antibacterial agents in some regions of the world, they are a relatively new technology in most countries, especially in this modern era. As such, phages are bound to elicit fear and myths as novel biocontrol substances. To clarify any misperceptions, all potential stakeholders must engage in public dialogue—from regulatory authorities and policymakers to farmers, merchants, health care practitioners, and people who might receive treatment. Workshops with relevant stakeholders could provide information regarding the potential benefits of phages, address concerns, and gain input on how phages could be utilized suitably within a local social setting. The criteria and processes by which phages would be certified as safe should be clearly communicated, and the ways they will be regulated and monitored must be explicitly defined. Facilitation of public discussion about phages can broaden acceptance of this new class of antibacterial products.

While there is great potential for phage applications, considerable work must be done before phages can be adopted in LMIC. It will be important to better understand how phages co-evolve with their bacterial hosts, to develop processes for regularly updating phage cocktails, and to optimize phage therapy regimens. In addition, safety assessments, regulatory procedures, and cultural buy-in will all be essential. Obtaining intellectual property can be difficult, especially on naturally occurring phages, so there is also a need to create commercial incentives other than patents to encourage companies to develop new phage products. Perhaps progress in this area will be motivated by both the immense public health needs, as well as by the tremendous size of developing country emerging markets. Ultimately, phage products have the potential to transform how infectious diseases are addressed in developing countries, helping to save lives as well as build economies.

AUTHOR CONTRIBUTIONS

TN conceived of the overall topic. TN, BC, DD, AE, EK, AM, and JP defined the contents of the manuscript and wrote the text. TN, AE, AM, and JP edited the manuscript. All authors read and approved the final manuscript.

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