ADVANCES IN PHAGE THERAPY: PRESENT CHALLENGES AND FUTURE PERSPECTIVES

EDITED BY: Andrzej Gorski, Petar Knezevic, Naomi Sulinger Hoyle and Shigenobu Matsuzaki

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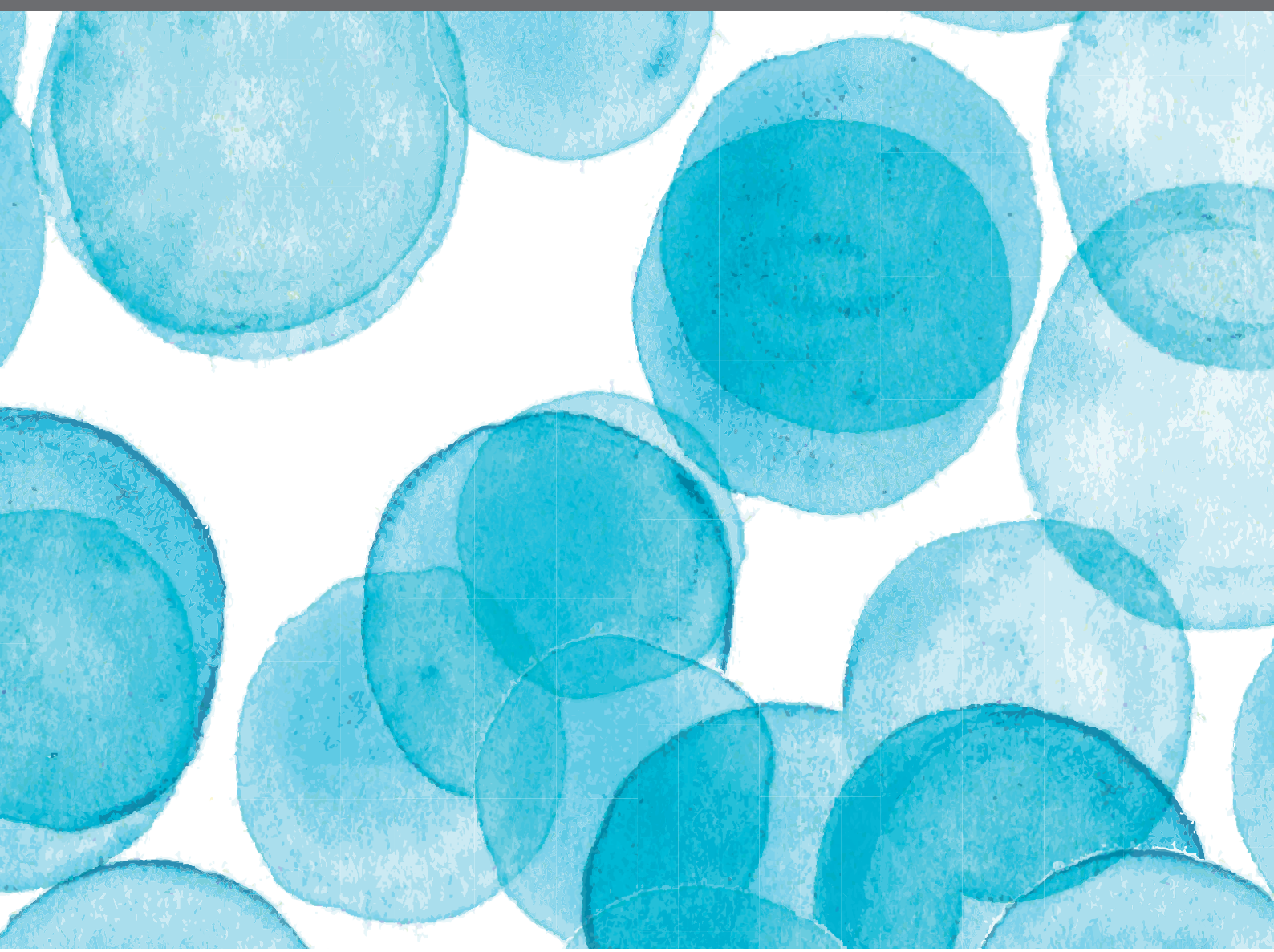

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ADVANCES IN PHAGE THERAPY: PRESENT CHALLENGES AND FUTURE PERSPECTIVES

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This Research Topic is dedicated to Prof. Elisabeth Kutter on the occasion of her 80th birthday. Dr. Kutter's career as a phage scientist has extended nearly 60 years. She has been a pioneer as a woman in science. She started to work with phage at the University of Rochester, New York working with Dr. Wiberg on radioisotopes making excellent progress in the field – progress which was even cited in Luria's 1969 Nobel Prize talk. Betty first encountered phage therapy during a visit to Georgia in 1990 which was part of a longer stay in the former Soviet Union under a US-USSR research exchange program. Dr. Kutter was one of the first Americans to advocate for phage therapy in the post antibiotic era.

Betty started hosting the Evergreen International Phage meetings in Olympia, Washington, from 1975 onward, which helped to develop a strong phage community with participation increasing over the years to 350 at the 23 rd biannual last year. Betty continues to be an active member in the phage community, sharing her experience and working with all of us toward her ultimate goal of making phage therapy available worldwide thus reducing the burden caused by antibiotic resistant bacterial infections.

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EDITORIAL
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Editorial: Advances in Phage Therapy: Present Challenges and Future Perspectives

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Editorial on the Research Topic

Advances in Phage Therapy: Present Challenges and Future Perspectives

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The emerging multiple- and pan-drug resistant bacterial strains enforce research in the field of new antimicrobial agents and therapeutic strategies, in the combat against life-threatening infections. In the post antibiotic era, bacteriophages have been considered as one of the major solutions to overcome the current medical crisis, offering many advantages over conventional antimicrobials. During the last two decades, a significant progress in phage therapy (PT) is evident, but we still need to identify and fill gaps in the knowledge, complement experience, provide additional proofs of PT efficacy and safety, as well as to reconsider methods and practical approaches to bring beneficial results for human health and well-being. The Research Topic *Advances in Phage Therapy: Present Challenges and Future Perspectives* encompasses the analysis of past experiences of phage application, current state of PT in contemporary medical practice, new original data and consideration of future perspectives (**Figure 1**).

EXPERIENCE IN PT

Since bacteriophage discovery, more than 100 years ago, there have been intentions to introduce phages into therapy for diseases for which bacteria have been etiological agents. When antibiotics were discovered, the aspirations of phage application in therapy were abandoned. However, in several countries, including Poland and Georgia, PT continued to be part of medical practice, to a lesser or greater extent. Today, beside a few completed and ongoing clinical trials, there are several phage therapy units throughout the world (e.g., USA, Belgium, Georgia, Poland, Australia), which continually provide valuable information. In the manuscript by Zaczek et al. the history of phage therapy in Poland was described in details, from the interwar period to the modern days and the establishment of the first Phage Therapy Unit at the Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław in 2005. The special value of this review paper is that it familiarizes us with numerous Polish scientists who have worked in this field but remained insufficiently known to the scientific community.

Khalid et al. reviewed phage application in bacterial foodborne diseases control (typhoid, cholera, shigellosis, *E. coli* caused diarrhea) and tuberculosis, indicating that this approach can be a solution for disease control in developing countries.

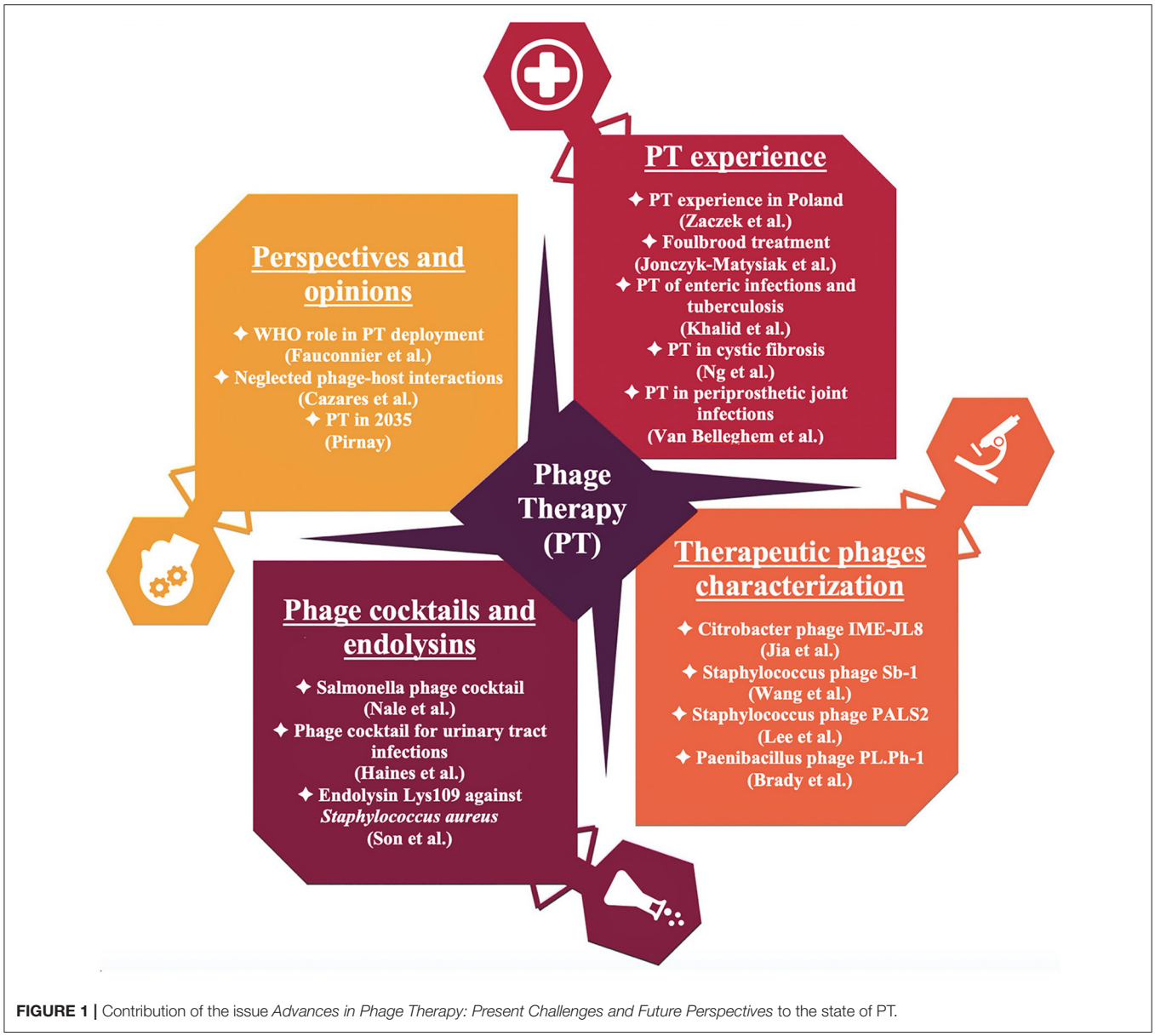

FIGURE 1 | Contribution of the issue *Advances in Phage Therapy: Present Challenges and Future Perspectives* to the state of PT.

In the article presented by Ng et al. phage application in the treatment of *Pseudomonas aeruginosa* infections in patients suffering from cystic fibrosis was reviewed, considering *in vitro* models, cell culture models, and future prospects. Another phage therapeutic application was considered in the review by Van Belleghem et al. who scrutinized major aspects of the prosthetic joint infection (JPI) pathogenesis and biofilm formation, phage properties and their interaction with immune system, their use as supportive therapy in JPI, as well as in prophylaxis.

Besides phage application as alternative remedy for bacterial diseases of humans, they have been considered in the context of beekeeping. Namely, American foulbrood, a disease caused by *Paenibacillus larvae* that infects honeybees (*Apis melifera*) can also be treated by bacteriophages. In the article by Jończyk-Matysiak et al., beside pathogenesis, epidemiology, diagnostics, conventional treatments and antibiotic resistance, particular attention has been paid to phenotypic and genotypic characteristics of *P. larvae* bacteriophages, their exploitation in PT, endolysin production, as well as the limitations of such approach to disease control.

THERAPEUTIC PHAGES CHARACTERIZATION

Within the framework of the scientific topic, several new phages were described. A new phage IME-JL8 (family *Siphoviridae*) of fish pathogen *Citrobacter freundii* was described in detail, including *in vitro* lytic efficacy and effect on biofilm. *In vivo* experiments demonstrated promising application of this phage for the prevention and treatment of *C. freundii* fish infections (Jia et al.). A novel jumbo phage PALS2 (family *Myoviridae*)

isolated from bird feces was characterized in detail and its anti-*S. aureus* potential was assessed (Lee et al.). Furthermore, phage Sb-1 (family *Herelleviridae*) was examined as a control agent against rifampin resistant *S. aureus* biofilm upon simultaneous or staggered addition of various relevant antibiotics (Wang et al.). The combination of Sb-1 phage and daptomycin was shown as the most promising in the context of exploitation of the phenomenon called "phage-chemical agent" or "phage-antibiotic" synergy. Related to the abovementioned foulbrood disease control, Brady et al. using state-of-the-art methods demonstrated that phage PL.Ph-1 does not adsorb only to vegetative cells of *P. larvae*, but also to bacterial endospores, which has significant applicative implications.

PHAGE COCKTAIL AND ENDOLYSIN DESIGN

Bacteriophages show group specificity to bacteria and one phage strain usually cannot infect all strains of one bacterial species. This issue can be overcome by preparation of phage cocktails that contain various phages infecting one species, with different lytic spectra. Haines et al. used ESBL-producing bacteria that cause urinary tract infections as a model to develop a useful method for phage selection and cocktail design, including Direct Spot Test, the Efficiency of Plating assay, the Planktoning Killing assay and the Biofilm assay. Examining 21 myovirus and one siphovirus, Nale et al. designed an optimal cocktail containing three carefully selected phages to combat various swine and poultry *Salmonella* serotypes. The cocktail potency was confirmed both *in vitro* and *in vivo*, using a co-infection and remedial regimen method on *Galleria mellonella* larva model. The authors confirmed the efficacy of the cocktail for treatment and prevention against *Salmonella* infection *in vivo*. Son et al. designed a chimeric endolysin Lys109 efficient against *Staphylococcus aureus*.

PERSPECTIVES AND OPINIONS

Cazares et al. presented an interesting opinion that phage ecology and evolution are usually neglected in phage therapy, particularly bacteria-phage interactions. They pointed out that quorum sensing (QS) can affect bacterial susceptibility to phages, while phage can modulate bacterial cooperation. Accordingly, the recommendation is to carefully examine these interactions to better exploit phage antibacterial properties.

As indicated, Khalid et al. pointed out potential of PT application in developing countries, and similarly was observed by Fauconnier et al. whose opinion is that PT use has been little explored for low-income and middle-income countries. The authors clearly declared that the World Health Organization (WHO) should have a prominent role in the deployment of phage therapy. For instance, the WHO could help promote the knowledge of PT and build a regulatory system for phage products through its vaccines prequalification (PQ) program.

Finally, Pirnay predicted PT destiny in the next 25 years, indicating the possibility for personalized therapies. The author described chain of events that can result in a cell free synthetic phage production—from community efforts, through support of health organizations to the implementation involving Artificial Intelligence and a Distributed Ledger Technology. Although it seems feasible in theory, Pirnay is aware that this is an ideal scenario which can be easily disturbed by many obstacles.

AUTHOR CONTRIBUTIONS

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

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frontiers in Microbiology

HYPOTHESIS AND THEORY
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Phage Therapy in the Year 2035

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The emergence of multidrug resistant bacteria in both community- and hospital-acquired infections is recognized as a major public health threat. Phage therapy is increasingly mediatized and researched as an additional tool for combatting antibiotic resistant infections. However, phages exhibit a number of properties that differ from antibiotics and hamper their development as pharmaceutical products and their application in therapy. This paper advocates a paradigm shift in the development and application of infectious disease therapeutics to cater for personalized phage therapy, which could be realized by the year 2035. More specifically, it presents a sustainable and ethical supply chain of instant synthetic phages, based on a community effort, supported and steered by public health organizations, and managed by a platform combining Artificial Intelligence (AI) and Distributed Ledger (DL) Technology.

Keywords: infectious diseases, antibiotic resistance, antimicrobial resistance, phage therapy, synthetic biology, artificial intelligence, machine learning, distributed ledger technology

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PREFACE

This paper offers a personal vision of what might be needed for phage therapy to finally break through as a mainstream antibacterial tool. It is influenced by historical and recent failures and uncertainties in the phage therapy field and aims at finding solutions based on future and emerging technologies that are supposed to model the science and society of tomorrow.

PHAGE THERAPY

Bacteriophages (phages) are the viruses of bacteria. Since time immemorial they have controlled the growth and spread of their bacterial hosts. Bacterial viruses are the most ubiquitous lifelike entities in our biosphere. There are an estimated 10 million-fold more viruses in the oceans than there are stars in the universe and if all the phages on Earth were stacked on top of another, this tower would stretch further than the nearest 60 galaxies (Suttle, 2013). They can easily be found wherever bacteria thrive: in sewers, rivers, or patients' urine and stool. Phages of human bacterial pathogens are most often composed of an icosahedral head, a sphere with 20 flat faces made of proteins and containing a nucleic acid genome, to which a protein tail is attached. When a strictly lytic phage adheres with its tail fibers and spikes to the surface of its target bacterium, the syringe-like tail sheath contracts and the tail core is driven through the bacterial cell wall, injecting the phage genome into the periplasm of the bacterial cell. Immediately, the bacterial DNA and protein synthesis machinery is hijacked to build copies of the phage. Some phages cut the bacterial DNA into pieces. After a latent period of minutes to hours, the newly formed phages burst out of their bacterial hosts, which are killed in the process. The phage progeny, which can run into the hundreds per bacterium, then go off to find new host bacteria to infect. As such, phages can be considered

as self-replicating antimicrobials. Importantly, phages have evolved to only infect certain target bacteria and are harmless to mammalian cells.

Early evidence of viral-like agents with antibacterial activity was reported by the English bacteriologist Frederick Twort, and by the French-Canadian microbiologist Felix d'Hérelle in 1915 and 1917, respectively (Sulakvelidze et al., 2001). In 1919, d'Hérelle exploited for the first time the therapeutic potential of phages when he used them to cure a boy suffering from dysentery in Paris. Phage therapy was immediately recognized as a therapeutic approach to treat bacterial infections and commercialization of phage therapy preparations was undertaken by several companies, such as L'Oréal in Europe and Eli Lilly Company in the United States (Sulakvelidze et al., 2001). In 1923, the Georgian microbiologist Giorgi Eliava founded the Eliava Institute in Tbilisi, Georgia, devoted to phage therapy research. It was the start of extensive phage therapy research and development in the former Soviet-Union. However, early uses of phage therapy were often unreliable and research into antibiotics had also been ongoing. The successful use of penicillin during the Second World War and its subsequent worldwide marketing led Western scientists to lose interest in phage therapy. Soviet researchers, in contrast, continued to develop phage therapy and to publish their results, but due to the Iron Curtain their knowledge and experience did not spread across the world (Sulakvelidze et al., 2001). At the dawn of the third millennium, the increasing health burden of infections with antibiotic resistant bacteria (Cassini et al., 2019) incited a renewed worldwide interest in phage therapy as a viable additional tool to the clinical management of bacterial infections (Thiel, 2004). All over the world, phage therapy centers are being set up, following in the footsteps of the Eliava Institute and the phage therapy unit at the Hirszfeld Institute in Wrocław, Poland (Miêdzybrodzki et al., 2012).

THE YEAR 2035

Fast forward to future Earth of 2035, a gloomy world characterized by human overpopulation, major ecosystem disruptions, global warming, and xenophobia.

While soaking in his bath, Dr. John Iverian, a retired microbiologist, suddenly felt an extremely painful sting in the back of his neck, followed by a sound like a small plane's propeller. He screened the environment and in the corner of his eye he saw a weird large insect with long creepy legs and antennas sitting on the wall next to his designer bathtub. Osuri, the home management system of Iverian's loft in the center of Antwerp, identified the insect as the brown marmorated stink bug *Halyomorpha halys*, which had spread across the world. Osuri's report, projected on one of the bathroom's video screens, mentioned that people, who were bitten, initially experienced a small red sore in the bite area of their skin. When left unattended, the bite wound would swell and produce puss. Tired and muzzy, non-chalant Iverian stepped out of his bath and went to bed. He had decided not to perform the elaborate wound treatment procedure, which had strongly been advised by Osuri. Early next morning, however, the bite had turned into a necrotic wound showing clear signs of infection.

Anxiously, Iverian activated his Phage-BEAM device. BEAM stood for "Bedside Energized Anti-Microbial." The device had the size and shape of a shoebox, but with a more elegant and polished look. The name of the device and its manufacturer were designed in colorful letters on the side of the seamless white enameled box. Iverian removed a swab from its sterile packaging and gently passed it over the entire area of the wound, making sure that the wound exudate thoroughly wetted the cotton wool tip of the swab. When the swab approached the "insert sample" area of the box, a tiny door opened as if magically, freeing a 10-inch high hologram of a lab technician, named Marcia. She showed Iverian where to dock the sample. Marcia was developed to guide the clients through the test procedure. "For best results, please insert a new phage bio-ink cartridge, Dr. Iverian," Marcia said. Just as it used to be for yesteryear's 2D printers, the cost of the bio-ink cartridges was almost as high as the cost of the Phage-BEAM device itself. According to "Business Insider," phage bio-ink was the second most expensive liquid on Earth, behind Chanel No. 8. Luckily, as one of the inventors, Iverian had obtained the right to always have the most recent version of this device at his disposition, including a continuous supply of reagents, for free.

Iverian knew perfectly how the device worked, so he did not need Marcia's help. First, DNA was extracted from the swab tip and the metagenome—all the genetic material present in the sample, including the infecting bacteria—was determined. Next, these genetic data were sent to a secured "Phage XChange" server where a complex AI-driven algorithm predicted the genome sequence of the phage that was most likely to lyze the infecting bacteria identified in the metagenome and was supposed to elicit the weakest immune reaction in the patient. The phage genome data were sent to the Phage-BEAM device, which first synthesized the phage genome and then the phage, using a proprietary bacterium-free phage production system.

Within 1 h after sampling, the device would produce a ready-to-use therapeutic phage product. Results of the step-by-step procedure would be transmitted to the enormous home video screen in Iverian's living room. Sitting in his LC2 armchair, listening to Mozart's Great Mass in C minor, Iverian anxiously waited for the results to come in. He had a bad feeling about this. The result sent shivers down Iverian's spine. Bacterial pathogen identified: *Streptococcus pyogenes* strain FE-2033! Osuri immediately activated the infection alert protocol, sending a message to the World Center for Disease Control and projected worrisome background information on the lethal flesh eating bacterial strain, which was considered an imminent threat to public health since 2033. For a moment, Iverian considered excising the infected wound and some surrounding healthy tissue with a kitchen knife, but he calmed down and decided to wait and apply the imminent Phage-BEAM product. An hour later, the Phage-BEAM device had produced synthetic phages. These phages were then mixed with the isolated bacteria, in a validation module, to test their *in vitro* efficacy. Fifteen minutes later, the green light was given for Iverian to commence treatment. Iverian applied the phages in a slow release hydrogel-based wound dressing, which had first been mixed with the concentrated

phage suspension produced by the Phage-BEAM device, and also contained synergistic antibiotics. The hydrogel temporarily relieved the pain, which further calmed him. Iverian repeated application of the phage and antibiotic-loaded hydrogel once a day. Wound infection improved within 24 h and after 7 days the wound was almost completely healed. Iverian's potentially life-threatening infection was successfully treated, timely and without leaving his home. But, for many previous decades, it had not been certain that phage therapy would break through to become a broadly applied and clinically useful antibacterial tool. The medical world had taken a while to realize that phage therapy did not need to be identical to antibiotic therapy, and this mainly because of the peculiarities of the active agents, the phages.

SOME RELEVANT PECULIARITIES OF PHAGES

Phages exhibit a number of properties that differ from antibiotics and hampered their development as pharmaceutical products and application in therapy. First, they tend to be specific about which bacteria they infect. They will at best target a considerable part of one single bacterial species, but at worst they will infect only a small number of strains within one species. Therapeutic phages can thus be selected to mainly kill one bacterial species, or a clinically relevant subgroup thereof, and spare the patient's beneficial bacteria (e.g., the gut, skin, or oral commensal flora). Most routinely employed antibiotics, in contrast, have a broad spectrum of activity, which can cause "collateral damage" to the patient's commensal microbiomes, which in turn can result in adverse effects such as the selection of antibiotic resistant bacterial species (e.g., *Clostridium difficile*) or antibiotic-associated diarrhea (Jernberg et al., 2010). The drawback of phage specificity is that the infecting bacteria need to be identified before starting phage therapy. In empirical antibiotic therapy, in contrast, broad-spectrum antibiotic cocktails that affect a multitude of Gram-positive and Gram-negative bacteria, and diverse fungi are typically used. When more information is known (e.g., from bacterial culture), treatment may consist of narrow-spectrum antibiotics, which more specifically target the bacteria or fungi identified to be causing disease.

Second, bacteria and phages are involved in a host–parasite relationship. Strictly lytic phages are ubiquitous in the environment and require the death of their bacterial host to complete their life cycle. Without hosts, phages cannot exist. Phages impose selection for resistant hosts, which in turn impose selection for effective phages. This results in what is called "antagonistic coevolution," an arms race between bacteria and phages, characterized by reciprocal evolution of bacterial resistance and phage infectivity (Buckling and Rainey, 2002). Just as with most antimicrobials, bacteria will thus also become resistant to phages (Luria and Delbrück, 1943; Schooley et al., 2017), but, in contrast to static antibiotics, phages have the capacity to overcome bacterial resistance (Buckling and Rainey, 2002). There are nevertheless indications that bacteria and phages will not indefinitely increase their respective resistance and infectivity (Fortuna et al., 2019).

PHAGE THERAPY APPROACHES

At the time of the phage therapy revival in the early 2000s, two distinct phage therapy approaches had been developed (Pirnay et al., 2011). In what could be called the *one-size-fits-all* approach, defined broad-spectrum phage cocktails, which were supposed to target the majority of bacteria suspected to cause certain infectious diseases, were applied. These predefined broad-spectrum phage cocktails were developed, produced, and tested within the current pharmaco-economic models, which had been designed to cater for "static" drugs such as antibiotics. However, truly broad-spectrum phage cocktails, active against most Gram-positive and/or Gram-negative bacteria commonly encountered in infectious diseases needed to contain large amounts of phages and turned out to be very difficult to develop. It was feasible to develop narrower spectrum phage cocktails, active against one or a few bacterial species, to be used in certain indications and minding that the infecting bacterial species were known in advance. For some bacterial species, such as *Staphylococcus aureus*, phages showing an exceptionally broad host range had been isolated and characterized (Vandersteegen et al., 2011). In PhagoBurn, a randomized controlled trial, success in achieving sustained reduction in *Pseudomonas aeruginosa* burdens in burn wounds was linked to initial susceptibility to the phage cocktail (Jault et al., 2019). However, one-third of the included patients were shown to harbor pre-existing *P. aeruginosa* strains resistant to the phage cocktail, which consisted of no less than 12 lytic *P. aeruginosa* phages. In addition, phage cocktails that were initially designed to be effective needed to be regularly updated (e.g., supplemented with new phages) in response to the emergence of phage resistance or the involvement of newly circulating clinically relevant strains. Finally, it was not known if confronting bacteria with high concentrations of fixed phage cocktails would cause the emergence, spread and persistence of bacterial phage resistance in hospitals and in the environment, similar to what had happened upon the massive use of antibiotics.

In *personalized* phage therapy concepts, one or more phages were selected from a phage bank, or from the environment, and possibly adapted (*in vitro* selection of phage mutants exhibiting increased infectivity) to more efficiently infect the bacteria isolated from the patient's infection site (Friman et al., 2016). Some phage therapy centers set up and maintained large therapeutic phage banks, which were regularly updated with new phages, widening and adapting the host range of the bank to the ever-changing bacterial populations. Personalized phage therapy approaches were potentially more sustainable, as only the infecting bacterium is targeted, resulting in less selection pressure toward bacterial phage resistance. However, they were also more elaborate and logistically complex than one-size-fits-all approaches, with bacterial strains and matching phages being sent all over the world (**Figure 1**). Moreover, precision medicine concepts were, in general, not compatible with most medicinal product (drugs in the United States) development and licensing pathways, which required several years and millions of euros (dollars) to complete, and this for every phage in the bank (Verbeken et al., 2012).

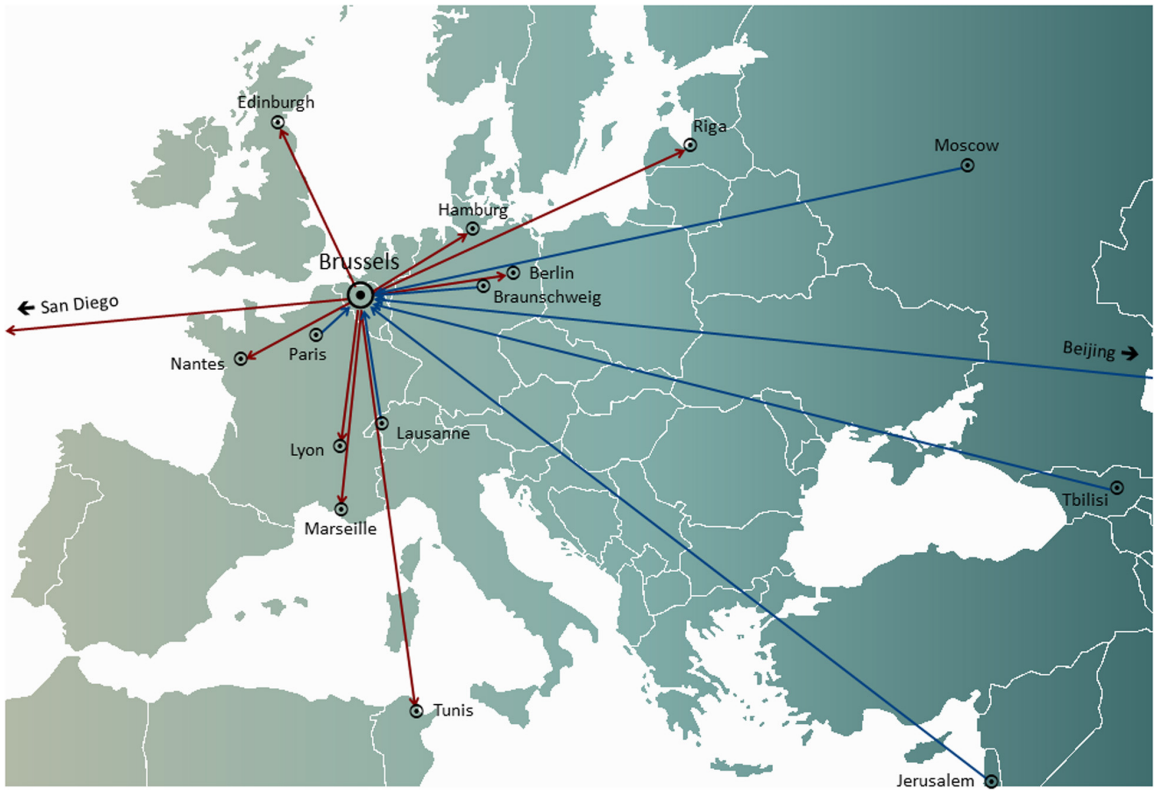

FIGURE 1 | International transfers of phages from (red arrows) and to (blue arrows) the Queen Astrid Military Hospital (QAMH) in Brussels in view of clinical applications over the period 2015–2020. On the national level, phages were dispatched from the QAMH to five university hospitals (not shown). In addition, the selection of matching phages often encompassed the transfer of the patients' bacterial isolates, and five international patients (two from France, two from the Netherlands, and one from Tunisia) were transferred to Brussels for phage therapy.

ENTER SYNTHETIC BIOLOGY

With the onset of the third millennium, synthetic biology approaches had been increasingly developed to reduce the specificity of phages and the emergence of bacterial phage resistance (e.g., structure-guided design) (Pires et al., 2016; Dunne et al., 2019). For instance, yeast-based platforms for phage tail fiber protein switching were elaborated to engineer hybrid phages with more predictable and extended host range (Ando et al., 2015; Yosef et al., 2017) and genetic engineering strategies (e.g., CRISPR-Cas editing tools) were developed to address other aspects such as negative patient-phage interactions (e.g., anti-phage immune response) (Brown et al., 2017), the potential emergence and spread of bacterial phage resistance mechanisms, and the release of harmful bacterial contents such as endotoxins (Hwang et al., 2018). Synthetic phage genomes needed to be rebooted to produce phage offspring (Barbu et al., 2016; Pires et al., 2016), through transformation of *Escherichia coli* or *Listeria monocytogenes* L-forms (Kilcher et al., 2018), or using cell-free transcription-translation (TXTL) systems (Rustad et al., 2018). Western regulatory frameworks had gradually started to cater for precision and personalized phage therapy approaches using naturally occurring phages (Pirnay et al., 2018), engineered phages (Dedrick et al., 2019), and synthetic phages.

The development of *ad hoc* and on-site therapeutic phage production devices, such as Phage-BEAM, did not run smoothly, Iverian recalled. To start with, it required artificial intelligence (AI)-based *in silico* phage matching and design. Deep learning (Martorell-Marugán et al., 2019), a subset of Machine Learning, was chosen to search for links between bacterial genomes and infecting phage genomes, because it was easier to scale to bigger number of samples. For instance, deep learning methods did not require so-called feature extraction, which would require gene/protein level annotation of phage and bacterial genomes and would limit predictions to certain known relationships between bacterial and phage features, such as phage tail fiber structures binding to specific bacterial cell wall receptors. As a down side, it needed to be powered by a continuous supply of massive amounts of data, linking lytic phage genomes to host bacterial genomes, and that's where the shoe pinched. Whole genome sequencing had slowly percolated into the practice of clinical microbiology (American Academy of Microbiology, 2016), but research institutes and pharmaceutical companies were not keen to submit their data to a single centralized database, and no investors were found willing and able to acquire the available data and/or to produce sufficient amounts of new data. A second obstacle that had to be overcome was the unavailability of quick, reliable, and affordable synthesis of

large DNA molecules. Initial DNA synthesis techniques were based on organic chemistry and produced relatively small DNA molecules. The *de novo* synthesis of phage genomes required assembling several genome fragments (Barbu et al., 2016; Pires et al., 2016; Lemire et al., 2018) in the yeast *Saccharomyces cerevisiae*, using yeast artificial chromosomes (Ando et al., 2015), or chemical assembly (Gibson et al., 2009). The development of a new technique to synthesize DNA, based on DNA-synthesizing enzymes found in cells of the immune system (Palluk et al., 2018), facilitated phage genome synthesis. Finally, some hurdles had to be overcome to develop generic cell-free phage production systems able to produce phages in high titers and exhibiting the same levels of bacterial infectivity as their natural analogs.

THE BREAKTHROUGH

The major problem was that it turned out to be very difficult to collect the massive amounts of linked phage and bacterial genome sequences necessary for the deep learning AI algorithms to predict and/or design phage sequences with a therapeutically acceptable level of accuracy. Iverian remembered that the real breakthrough came when the not-for-profit organization "Phage

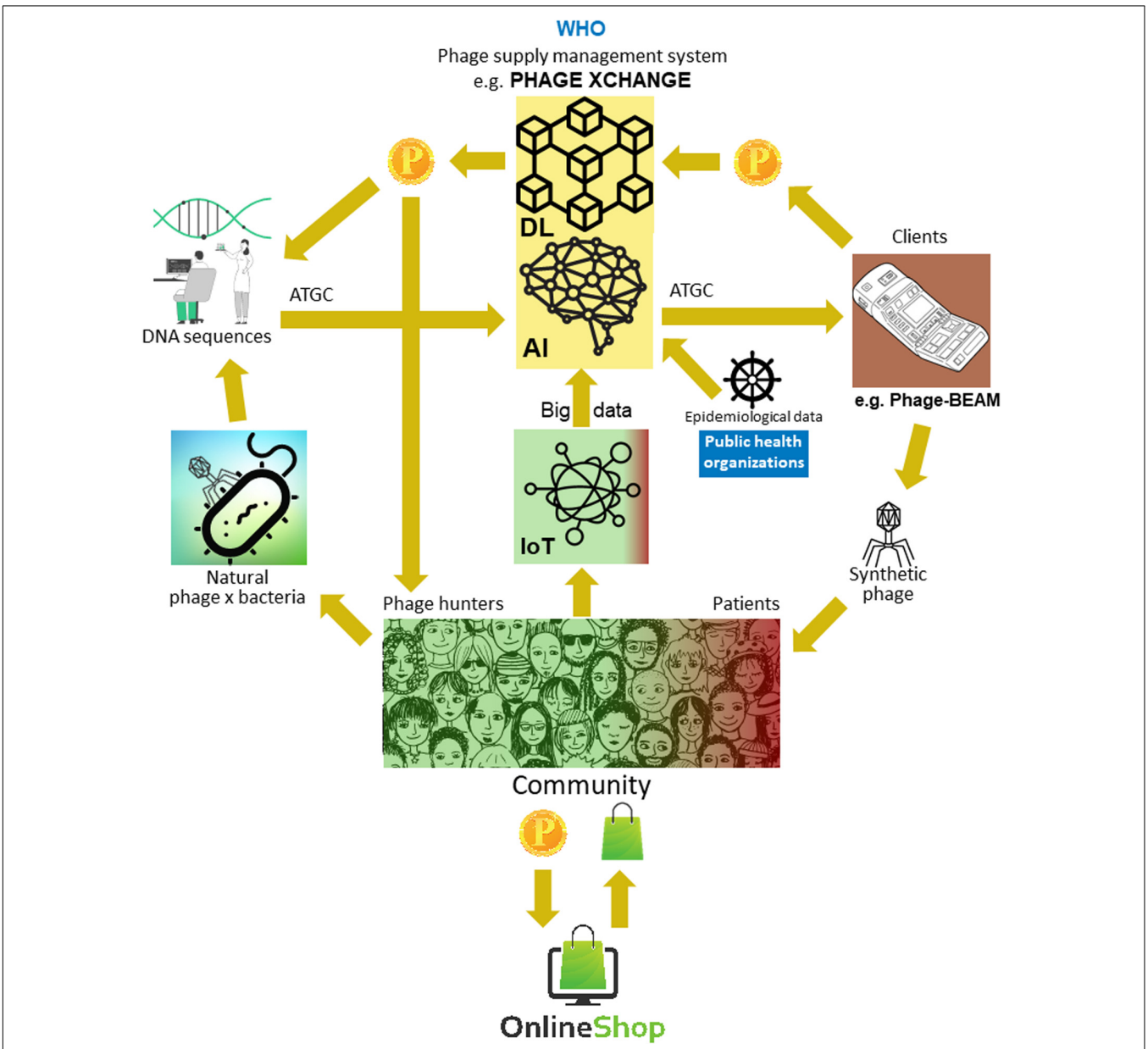

FIGURE 2 | Vision of how the phage supply chain might be organized in 2035. AI, artificial intelligence; ATGC, DNA sequence; BEAM, bedside energized anti-microbial; DL, distributed ledger; IoT, Internet of Things; P, PhageCoin; WHO, World Health Organization.

XChange" launched its global phage governance platform of the same name to create an efficient, standardized, sustainable, and ethical phage supply chain (**Figure 2**). Phage XChange mainly consisted of an AI module and a Distributed Ledger (DL) (Thiebes et al., 2020). The platform's AI module analyzed linked phage and bacterial genomes to predict and design potent phages for clients. It also predicted which bacterial pathogens needed the most urgent attention, based on the Internet of Things (IoT) and Big Data and information provided by international public health organizations, such as the World Health Organization (WHO) and national Centers for Disease Control. These data steered the system toward the isolation and characterization of the most urgently needed phages.

The platform's DL module ensured a sufficient, qualitative, and recorded input of linked phage/bacterial genome sequences to the AI module and ditto supply of phage sequences to clients, in compliance with the provisions of the Nagoya protocol (Expert round table on acceptance and re-implementation of bacteriophage therapy et al., 2018). The DL immutably recorded all stakeholder (e.g., suppliers of data, sequencing services, and clients), transaction, and contract details. It also recorded the exact quality, specifications, and weight of the supplied material. An algorithm determined the non-redundancy and estimated the weight (e.g., the virulence and host range of the phages) and desirability of the submitted material. Phages targeting emerging bacterial pathogens were of course most wanted. Most patent issues were obviated. The DL acted as a payment ledger to assure that all parties were paid timely and fairly. A number of PhageCoins (the platform's crypto currency) were attributed to the suppliers in relation to the quality, weight, and desirability of the supplied material. Clients extracting prediction results (phage sequences) through the DL paid an amount of PhageCoins, proportionate to the estimated value of the phages. These PhageCoins were used to maintain the DL, to assure a sufficient and continuous inflow of material, and to expand phage virulence and host range data (matching phages to bacteria). An additional injection of funds and incentive to supply material was found in producers and suppliers of all kinds of goods. With the instantly earned PhageCoins, phage suppliers could buy online all kinds of products at strongly reduced prices, from laboratory- and school equipment to sports items. These goods were provided through corporate sponsorship. Several established companies sponsored PhageXchange in exchange of tax reductions, publicity, and the image of a socially responsible brand. The weight of the supplied material, and thus also its value, were initially undervalued, but were re-evaluated at regular intervals (iteration) and suppliers were attributed more PhageCoins when warranted. Even though useful from the moment it was introduced, the platform only became really successful when it was put under the protection of the WHO, in analogy to the worldwide system of traceability, transparency, vigilance, and surveillance of Medicinal Products of Human Origin (Warwick et al., 2013). A formal agreement between Phage XChange and the WHO increased international confidence in the long-term sustainability of the platform and protection from unethical commercial exploitation. The search for potent therapeutic phages soon became a community effort aimed at solving the antibiotic resistance crisis, with independent "phage hunters," schools, scout groups, villages on the banks of the Amazon River, etc., isolating and submitting phages to Phage XChange, in exchange of PhageCoins. At the margins of this, various companies and institutions developed peripheral equipment and services, such as phage isolation kits and sequencing and phage synthesizing platforms (e.g., the PhageBEAM device). In anticipation of these devices, intermediary solutions were offered, whereby the phages themselves were obtained through the DL.

EPILOG

This view on the future of phage therapy provides an optimistic ending to the antibiotic resistance crisis. The *ad hoc* and on site production of synthetic phages, linked to a global, community-based, phage management system, turned out to be a welcome and affordable (for social security systems) extra weapon in the fight against antibiotic resistant bacterial infections. However, it was not a magic bullet; it was a synergistic supplement to established antimicrobials. The instant and cell-free production of synthetic phages, whether designed or not, had considerable advantages over classically produced (in bacterial hosts) natural phages:

(i) There was no need to maintain physical therapeutic phage banks and to dispatch the patient's bacterial isolates and the matching therapeutic phages all over the world.
(ii) Synthetic phages against bacteria causing eminent public health threats, such as the 2011 *E. coli* O104:H4 outbreak in Germany (Merabishvili et al., 2012), or bacteria (suspected to be) used for bioterrorism (Joñczyk-Matysiak et al., 2014) could be timely produced on site.
(iii) Phages against bacteria causing potentially lethal diseases, for which no non-lethal production host strains were available and whose propagation used to require biosafety level-3 (bsl-3) bio-containment precautions, could be synthesized in bsl-1 conditions.
(iv) When no phages could be isolated from sampling sites, for instance, because the bacterial host strains used in the isolation techniques were not susceptible to the desired phages, (predicted) phage genomic sequences, extracted from metagenomic data (Reyes et al., 2010; Amgarten et al., 2018), could be used to produce synthetic phages.
(v) Synthetic phage preparations contained no (or smaller amounts of) molecules that could have a negative impact on patients (e.g., endotoxins).
(vi) Devices were adapted to produce synthetic phages during extended space travel and space colonization (Taylor and Sommer, 2005).

There is little chance that these predictions will come true. It is probably too shortsighted to think that a community-based effort, supported by public health organizations and managed by a global, sustainable and ethical platform, could be at the heart of a solution to the current worldwide antibiotic resistance crisis.

Some parts of the proposed system, such as cell free production of synthetic phages using a bedside device, have a reasonable chance of being realized, while other elements, such as corporate sponsorship, will likely remain limited to the realm of science fiction. You may say that I'm a dreamer, so feel free to wake me up in 2035 to confront me with reality!

AUTHOR CONTRIBUTIONS

J-PP conceived the vision and drafted the manuscript.

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REVIEW
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Phage Therapy in Poland – a Centennial Journey to the First Ethically Approved Treatment Facility in Europe

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Although phage discovery is an unquestionable merit of the English bacteriologist Frederick W. Twort and the Canadian–French microbiologist Félix d'Hérelle, who both discovered phages over 100 years ago, the Polish history of phage studies also dates back to those years. In contrast to the Western world, developing phage treatment in Poland has never been abandoned despite the country's tense history marked by the Second World War (WWII) and the communism era. Today, Poland takes a prominent and remarkable place in the phage research area. Furthermore, established in 2005, the Phage Therapy Unit at the Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, the first such center within European borders, has quickly become a model for other centers in the world facing the issue of widespread antibiotic resistance. This article constitutes an attempt to fill the gap in the scientific literature by providing a comprehensive summary of the long tradition of phage research in Poland.

Keywords: phage therapy, phage discovery, phage research, Hirszfeld Institute, Phage Therapy Unit, Polish history

INTRODUCTION

Recent years have shown a remarkable boost in the development of phage treatment across the globe. The growth of antibiotic resistance sparked a new debate on the problem that has been known for decades and changed the way in which novel therapies are perceived by the public. Such a status of phage therapy did not always seem to be the case. In the first years following phage discovery, phages rapidly lost their initial potential for antibiotics. The success of penicillin postponed the resurgence of phage interest until the next century. The outbreak of the Second World War (WWII) increased the value of penicillin. At that time, the major goal was to have an adequate supply of the drug. Production of penicillin in the United States alone jumped from 21 billion units in 1943 to more than 6.8 trillion units in 1945. As of March 1945, penicillin was widely available at pharmacies across the States (Aldridge and Sturchio, 1999). Hence, one may be surprised that phages had been used in medicine 10 years prior to the discovery of penicillin. However, not all countries rejected phages in favor of penicillin. In such a way, Eastern Europe became the only part of the world where phage therapy was considered a serious alternative to

antibiotics. Even just a few years ago, the "phage therapy" term was still barely noticeable in PubMed search results, indicating that this resurgence is still an ongoing matter (Pirnay et al., 2019).

Early research on phage, which was by definition cheaper and limited to basic microbiological assays, constituted a great opportunity for the less wealthy and less developed part of Europe. Surely, that opportunity has been fully realized. Among the Communist states, Poland and Soviet republics (particularly the Georgian and Russian SSR) are best known for their significant contribution to the development of phage treatment. On the other hand, in the West, inadequate and poor methodology contributed to the perception of phages as a failed concept. Such discrepancies between the Western and Eastern parts of Europe are probably the best explanation as to why countries on the east side of the iron curtain have become pioneers in research on phages and their therapeutic connotations.

Since 2005, a year after Polish accession to the European Union, phage therapy in Poland has changed dramatically becoming a model way of treatment for other centers in the world (Gill and Young, 2011). The establishment of the Phage Therapy Unit (PTU) at the Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland, coincided with a sharp increase in morbidity and deaths in the world caused by antibiotic-resistant superbugs. The beginning of the 21st century left little illusions regarding the future of antibiotics. The danger of multidrug-resistant (MDR) strains has even been emphatically entitled the "Battle against antibiotic resistance is being lost" in the pages of *Lancet* (Morris, 2007).

Over the years, the phage center at the Hirszfeld Institute has been highlighted by numerous prestigious publications, such as Science or Nature Medicine (Stone, 2002; Hausler, 2006). Hausler described vivid examples of patients who could officially seek a phage treatment at Wrocław clinic years before it became a popular subject in the Western mainstream media. In 2004, the leading national German daily newspaper *Die Welt* published an article about Polish phage therapy conducted at the Hirszfeld Institute entitled "*Viren als Verbündete gegen Infektionen*" [Viruses as allies against infections] with the conclusion that "Poland has taken on the international leadership in clinical phage therapy in the last decades" (Bettge, 2004). These efforts cannot be underestimated in the era of rapidly growing phage biotech startups that call themselves "clinical-stage" companies despite their little scientific background, experience in phage treatment in humans, and no significant achievements. Keen, in his article summarizing a century of phage research, predicts that in the future phage-based therapies will become widely used in medicine and agriculture (Keen, 2015).

However, in the following sections, we will focus solely on the past and try to present the rich and fruitful centennial history of phage therapy on Polish soil, a story that dates back to regaining independence by Poland after 123 years of its partition and loss of independence. We believe that Polish accomplishments in this field deserve proper attention in the history of phage research and therapy.

THE INTERWAR PERIOD

The first article on phage therapy was published in French in 1921 and was focused on staphylococcal skin infections (Bruynoghe and Maisin, 1921). At that time, Poland was a young independent state trying to re-establish its statehood in a new reality after the First World War (WWI) and after 123 years of being wiped off the map of Europe. The Second Polish Republic was the sixth largest country in Europe inhabited by nearly 30 million people. In contrast to the Soviet Union, shortly after WWI, Poland's significance in the world was rather limited. Thus, one can safely say that the beginnings of the Eliava Institute in Tbilisi, Georgia, which was founded in 1923, were quite different from the situation of Polish scientists. Institute Pasteur served as a model for building Soviet bacteriology. Furthermore, an extraordinary role in the development of the Eliava Institute was played by famous scientist Felix d'Hérelle. The founder of Georgian Institute, Prof. George Eliava worked with d'Hérelle in Paris in the years 1918–1920. Thanks to that relationship, Eliava had direct access to ground-breaking phage work (Myelnikov, 2018). In short, Polish microbiologists could not compete with a well-prospering Eliava Institute. The Institute, during its best times, employed approximately 1,200 researchers and support personnel and produced phage preparations (often several tons a day) against a dozen bacterial pathogens (Sulakvelidze et al., 2001). The tables were to turn almost a century later when Poland joined the European Union in 2004. Despite such inequalities in the situation of both countries, it would be a mistake assuming that Polish achievements in the phage field were meaningless. The first Polish work on phage treatment in controlling dysentery was published by the medical periodical *Lekarz Wojskowy* [Military Physician] in 1923 (Kalinowski and Czyż, 1923). It was just a few years after d'Hérelle's pioneer attempt to treat dysentery with phage in 1919 (Dublanchet and Fruciano, 2008).

The Polish microbiologist Prof. Władysław Kunicki-Goldfinger sheds more light on Polish phage scientists who actively published their articles in the interwar period (Kunicki-Goldfinger, 1992). He recognizes such names as Fejgin, Lipska, Łomiński, Sierakowski, and Szymanowski, among others. Particularly, Dr. Irena Lipska deserves further attention as her work has been cited by others several years after her publishing activity (Kennedy et al., 1986; Hsu et al., 2002). In 1931, she presented her findings at the 9th International Dairy Congress in Copenhagen, Denmark. Her abstract entitled "d'Hérelle phenomenon in milk" focuses on phage presence in milk and their possible antibacterial action in it, a phenomenon that is still of high importance among scientists (Lipska, 1931). She continued her research in the following years (Lipska, 1937). Dr. Lipska was also responsible for supplying the Ujazdowski Hospital in Warsaw with phage preparations that were later used during WWII in the years 1940–1944 (Letkiewicz et al., 2017).

Bronisława Fejgin

The outbreak of WWII brought a real tragedy into the lives of all Poles and the scientific community was no exception. Balińska and Schneider in a translation of Prof. Ludwik Hirszfeld's autobiography entitled "Ludwik Hirszfeld: The Story of One

Life" quote Hirszfeld's memories from that time (Balińska and Schneider, 2010). Prof. Hirszfeld lists the names of several Polish bacteriologists who died, were tortured, or were killed during WWII. Among many others, the name of Bronisława Fejgin is listed, who was a member of the team responsible for developing an outstanding method of testing water for the presence of paratyphoid germs. It is not a well-known fact that one of the first Polish references to phages comes from her. Bronisława Fejgin was a Polish-born Jewish physician. In 1914, she graduated from Sorbonne Medical School in Paris. Thus, most of her work was published in French (Fejgin, 1923, 1936; Fejgin et al., 1927). Her legacy in the field of bacteriology and serology is of ongoing relevance (Weisz and Grzybowski, 2016). In 1926, Bronisława Fejgin developed the laboratory typhus identification method which was later clinically implemented by Ludwik Fleck in 1942 in the Lwów Ghetto (Weisz and Grzybowski, 2011, 2016). The importance of her achievements in the phage field was already recognized in 1928 by Klosterman and Small (1928). The authors mentioned her name in the first sentence of their publication along with such prominent names like d'Hérelle and Blair to emphasize her success in isolating a lytic phage against bacteria classified then as *Bacillus diphtheriae* (today *Corynebacterium diphtheriae*). What might be even more unheard for today's phage researchers, Weisz and Grzybowski suggest that the discovery of phage by Twort and d'Hérelle was, at least partially, based on Fejgin's preliminary research as she described a lytic agent that would later become known as a phage. At this point, it must be added that she never claimed such achievement for herself and fully respected d'Hérelle's pioneer discovery. In 1927, in her article published entirely in Polish she describes "d'Hérelle's phenomenon" as the main driving force responsible for bacterial lysis (Fejgin, 1927). In this article, she predicted with great accuracy the future of phage research, concluding that "facts related to the discovery of invisible bacterial forms (. . .) are ready to shake faith in the immutability of bacterial species." Her legacy is also unique for another reason. Back then, the scientific world was mostly dominated by male researchers. These two facts considered together lead two the obvious conclusion that Bronisława Fejgin deserves a significant recognition from not only phage researchers but also the entire scientific community. There is no doubt her achievements would have been even more significant if not for her tragic death in the Warsaw Ghetto in January of 1943. Currently, it is very hard to find her complete scientific work in the form of publications but Fejgin appeared repeatedly in articles after WWII. For instance, Coetzee and Sacks emphasize in the prestigious journal Nature her role in detecting a lysogenic strain of *Proteus* (Coetzee and Sacks, 1959).

Jerzy Jasieński

Around the same period, Dr. Jerzy Jasieński from Jagiellonian University in Kraków used phage lysates to combat staphylococcal purulent infections in a group of 40 patients. He revealed his results in 1927 in the pages of *Polska Gazeta Lekarska* [Polish Medical Journal] entitled "*Próby zastosowania bakteriofagji w chirurgji*" [Attempts to use bacteriophagy in surgery] (Jasieński, 1927b). In his research, 75 out of 85 collected *Staphylococcus* strains were sensitive to phages that had been isolated by him from patients' infection sites. However, Jasieński was skeptical in terms of subcutaneously injected *Staphylococcus aureus* phage due to the unsatisfactory clinical outcome of applied treatment (among 11 patients with bone infections and chronic furunculosis only two cases indicated full recovery). Although his methodology does not meet today's standards, he recognized back then that phage preparations are generally safe for patients with no evident correlation between clinical outcome of phage treatment and the response of patients' immune system as reflected by anti-phage antibodies. The work described above is not the only article Jasieński published in 1927. His review paper from the same year entitled "*O Bakteriofagji*" [About Bacteriophagy] constitutes a remarkably comprehensive and detailed summary of the then state of knowledge about phage therapy in the interwar period (Jasieński, 1927a). On 30 pages, the results of phage authorities such as d'Hérelle, Gratia, Arkwright, Preisz, and Eliava were in detail evaluated and described by Jasieński. In the summary, he concludes that *in vitro* results obtained in the laboratory setting should not be directly translated into the human model. It is hard not to notice that such rules of evidence-based medicine (EBM) are important especially nowadays when articles determining the favorable outcome of phage treatment based only on *in vitro* tests or animal models are being published extensively almost on a daily basis.

THE POSTWAR PERIOD

The atrocities of WWII do not need closer attention here as this subject has been visited countless times and is still vivid in the memory of many people. Prof. Ludwik Hirszfeld in his autobiography writes about "immensely difficult circumstances" under which Polish scientists were forced to work in postwar Poland (Balińska and Schneider, 2010).

Despite such historic turbulences, phage treatment was still conducted by Polish physicians. An interesting example can be found in *Dzienniki Powojenne* [Post-war Diaries] by the famous Polish writer Maria Dąbrowska, published posthumously in 1996 (Dąbrowska, 1996). This personal collection of writer's memories provides a unique insight into a rough Polish history in the 20th century. The author mentions about her struggles with a purulent pelvic inflammatory disease that lasted for 4 months in 1942. It was then that she was treated by phages against *Escherichia coli*. Citing her own words, "Dr. Czubalski cured me (of the disease) starting from a four-time blood transfusion and then by applying phage provided by Dr. Kryńska along with his own methods of eliminating inflammation caused by *E. coli*." She adds that Dr. Czubalski literally saved her life when other doctors from the university clinic had given up on her. Unfortunately, Maria Dąbrowska does not provide any more details about the therapeutic use of phages, but even this short section constitutes an important contribution to the whole picture of Polish medicine with phage as a recognized method of fighting bacterial infections in the most severe cases. It is worth mentioning that Maria Dąbrowska was already quite famous back then and the use of phages could be an expression of the highest concern for the patient.

During the period following the end of WWII, research on the therapeutic application of phages continued. In 1951, Szczepańska on the pages of *Pediatria Polska* [Polish Journal of Pediatrics] published her preliminary results on the preventive application of phages in the newborns (Szczepańska, 1951). In the same journal, Lipska published her article in which she described her experience with phage therapy in infants during the war and early postwar period (Lipska, 1951). We learn from this article that at least 100 Polish physicians applied phages during the German occupation, when other antibacterials (i.e., sulfonamides) were not available. Certainly, she was responsible for the introduction of this treatment method in pediatric health care centers in postwar Warsaw. In the subsequent years, Dr. Irena Lipska was involved in the use of phage therapy in patients with typhoid infections.

Interestingly, recently published review on the history and development of phage therapy in Brazil describes work of aforementioned Dr. Irena Lipska (De Freitas Almeida and Sundberg, 2020). The authors cite her article published during the postwar period. In 1950, Lipska published a recommendation to the Brazilian medical community on the prophylactic use of phages to protect newborns against diarrhea (Lipska, 1950). Given as the first liquid after birth, phages were found to be "simple and harmless." The article was published in Portuguese. In June 1950, Lipska participated in the international conference in Rio de Janeiro, so the publication in Portuguese is probably related to this event.

Ludwik Hirszfeld

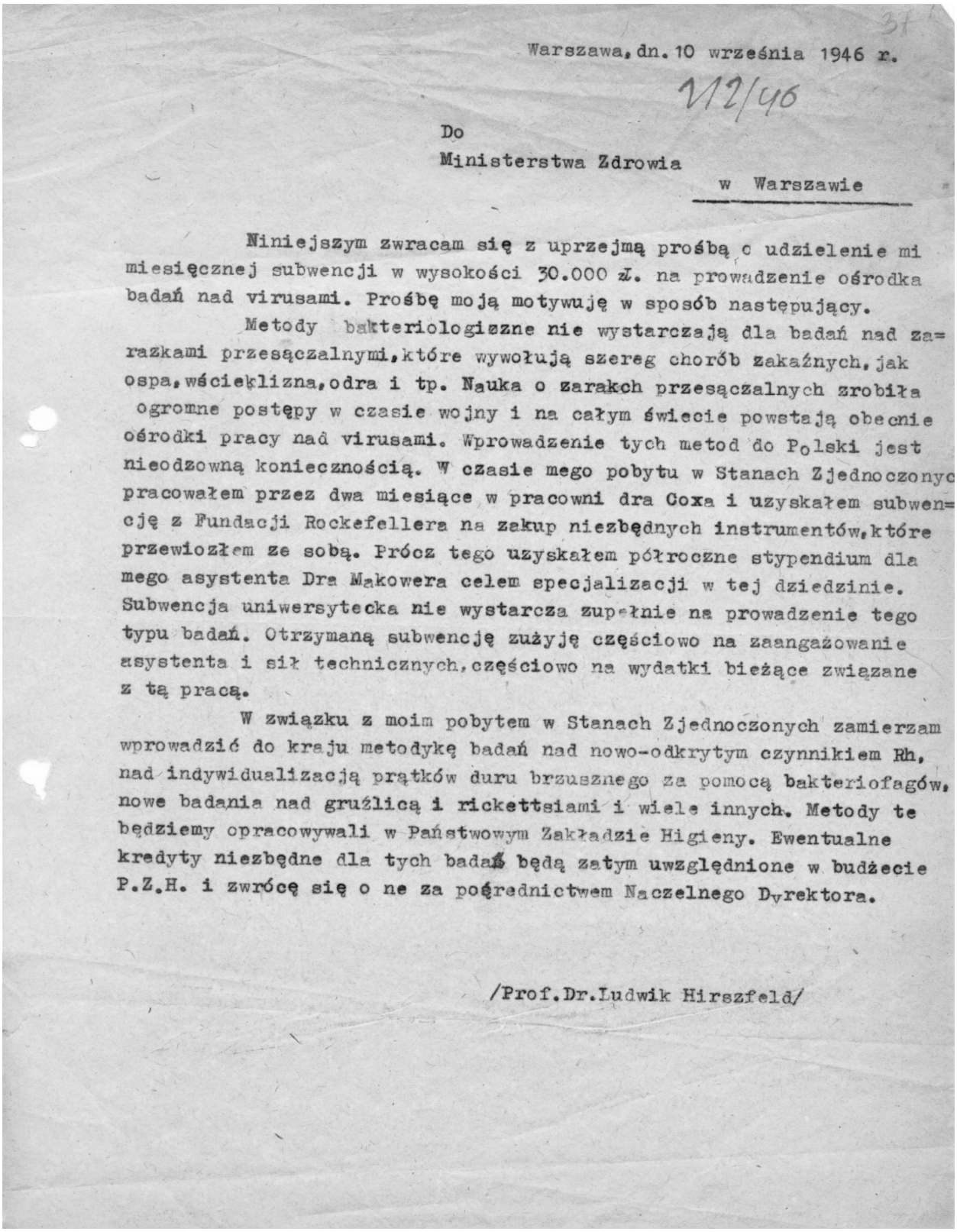
When analyzing phage research on Polish soil in the postwar period, it is impossible not to mention Prof. Ludwik Hirszfeld. His indisputable achievements include naming of the blood groups (A, B, AB, and 0) along with the discovery of their heritability and the discovery of serological conflict between mother and child, which was later confirmed by the presence of the Rhesus (Rh) factor. Hirszfeld was an honorary doctor of the universities of Prague and Zurich and in 1950 was a candidate for the Nobel Prize, which he ultimately did not receive, probably for solely political reasons (Kucharz et al., 2010). The '50s of the XX century was a period of the highest intensification of the so-called Cold War, which may have affected the decision of the Nobel Committee (Kozuschek, 2005). Obviously, these are not all of Hirszfeld's achievements. During WWI, he conducted vaccination programs and fought the epidemics of spotted fever, typhoid fever, and malaria in the south of Europe. Over this period, Hirszfeld isolated a strain of typhus bacillus that was later named to honor him as *Salmonella hirszfeldi*. He found out that the strain was an important etiological agent of typhoid, and he considered that it might have been the cause of the epidemics (Lonc and Gościniak, 2012).

However, for the phage community, the most crucial research seems to be Hirszfeld's research on phages he conducted right after WWII. Contrary to Bronisława Fejgin, Hirszfeld together with his wife Hanna and daughter Maria survived the Warsaw Ghetto, which they left in 1942. After the tragic death of their daughter in 1943, both spouses lived in secret in the village Wesoła near Warsaw until the end of the war (Jasińska, 2014).

On August 1, 1945, Hirszfeld went to Wrocław, a city located within the newly created Western borders of Poland. He was involved in organizing the first Faculty of Medicine, which was established in the same year as one of the six faculties of the University of Wrocław and Technical University. He also established the Department of Medical Microbiology, which was later transformed into the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences. An invaluable source of Hirszfeld's activity in the first years after arriving in Wrocław constitutes his extensive correspondence kept in the archives of the Hirszfeld Institute. The collection includes numerous letters written by and to Hirszfeld. From that correspondence, we learn that in September 1946, roughly 1 year after the end of WWII, Hirszfeld applied to the Ministry of Health for funding amounting to 30,000 Polish Zlotys. He motivated his request with the necessity of conducting research on phage against typhoid fever pathogens among others that could be conducted at the PZH [*Państwowy Zakład Higieny* (State Department of Hygiene)] (**Figure 1**).

In the same year, Hirszfeld visited Prof. James Craigie in Toronto. Hirszfeld was extremely impressed by his work on phage. He noted phage narrow specificity against bacterial typhoid pathogens and praised the significance of such a phenomenon for medicine (Balińska and Schneider, 2010). He returned from this trip with selected phages and their bacterial hosts with the intention of passing them to individual branches of the PZH. In a letter to the Director of PZH, Prof. Feliks Przesmycki, dated June 1947, Hirszfeld informed him that the phages he brought to Poland had been propagated and he planned to organize an additional phage typing course later that year. Hirszfeld continued his work on phage in the Wrocław branch of the PZH, which he described in August 1947 in a letter to Dr. Zygmunt Grynberg, the Director of the Organizational Department of the Ministry of Health. We learn from that letter that methodology regarding phage typing against typhoid fever bacteria had been developed by the Hirszfeld team. Furthermore, Hirszfeld announced that the International Committee in Copenhagen had chosen the Wrocław branch of the PZH as the national phage research center in July 1947.

Ludwik Hirszfeld played a leading role in the initiation of phage therapy in Poland after WWII. In a letter to Hirszfeld dated 22 April 1948, Dr. Zdzisław Przybyłkiewicz, then the director of the Medical Microbiology Institute at the Jagiellonian University in Kraków, expresses his gratitude for allowing him to use phages for therapy and points to the difficulties in obtaining feedback on the results of phage treatment carried out in different hospitals. In view of those difficulties, Przybyłkiewicz suggested that the therapy could be continued at the infectious ward of the St. Lazarus Hospital in Kraków using personalized phages prepared by his team (**Figure 2**). This approach could strengthen efforts to obtain reliable data on the safety and relative efficacy of phage therapy. Regretfully, we were unable to identify any publications related to this effort and the untimely death of Hirszfeld caused a temporary regress in further developments of phage therapy. At any rate, this incident confirms that Hirszfeld tried to expand phage therapy to other Polish cities, while the role of Dr. Zdzisław Przybyłkiewicz in the promotion of that therapy also needs to



Warszawa, dn. 10 września 1946 r.

212/46

Do
Ministerstwa Zdrowia
w Warszawie

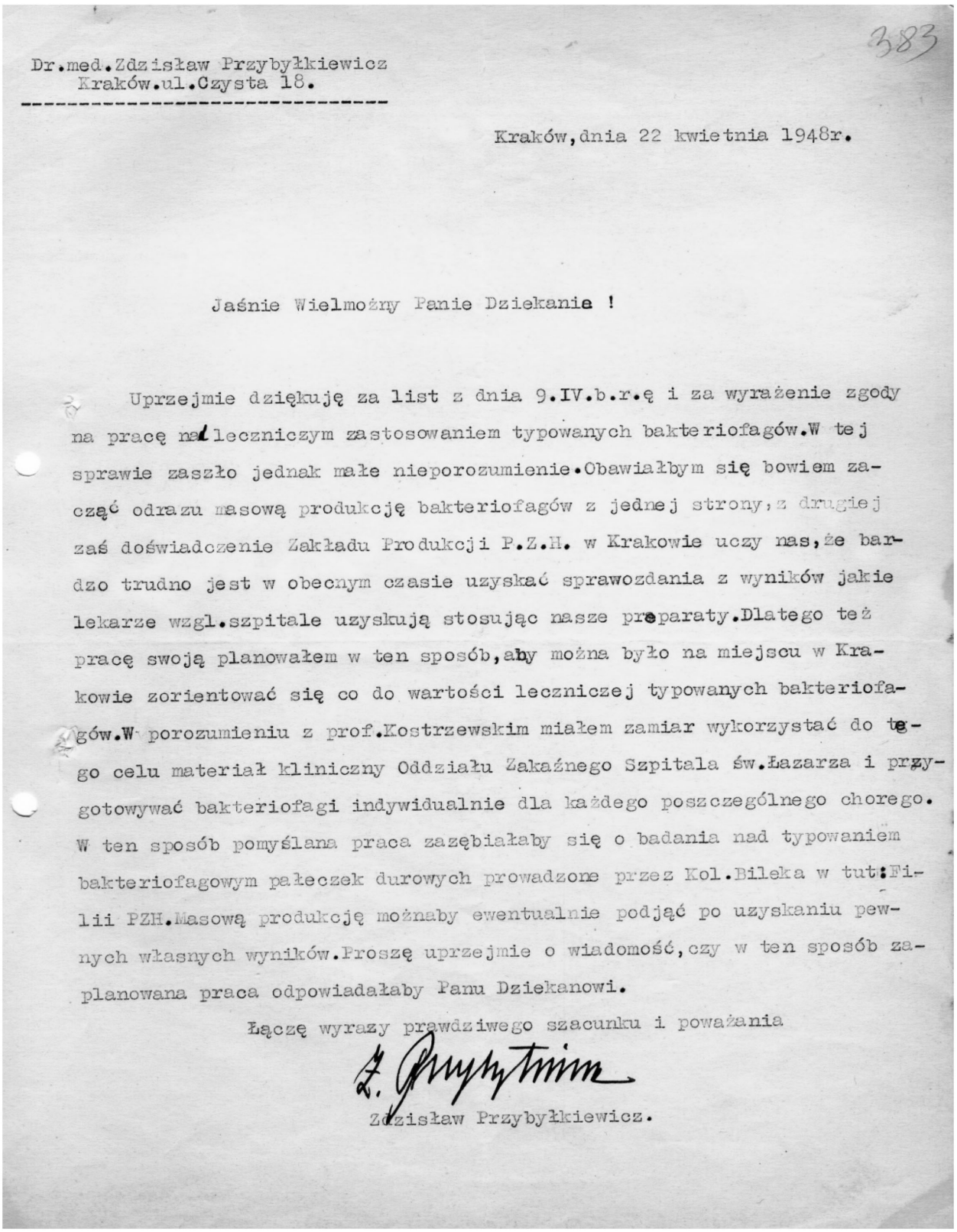
Niniejszym zwracam się z uprzejmą prośbą o udzielenie mi miesięcznej subwencji w wysokości 30.000 zł. na prowadzenie ośrodka badań nad virusami. Prośbę moją motywuję w sposób następujący.

Metody bakteriologiczne nie wystarczają dla badań nad zarazkami przesączalnymi, które wywołują szereg chorób zakaźnych, jak ospa, wścieklizna, odra i tp. Nauka o zarakch przesączalnych zrobiła ogromne postępy w czasie wojny i na całym świecie powstają obecnie ośrodki pracy nad virusami. Wprowadzenie tych metod do Polski jest nieodzowną koniecznością. W czasie mego pobytu w Stanach Zjednoczonyc pracowałem przez dwa miesiące w pracowni dra Coxa i uzyskałem subwencję z Fundacji Rockefellera na zakup niezbędnych instrumentów, które przewiozłem ze sobą. Prócz tego uzyskałem półroczne stypendium dla mego asystenta Dra Makowera celem specjalizacji w tej dziedzinie. Subwencja uniwersytecka nie wystarcza zupełnie na prowadzenie tego typu badań. Otrzymaną subwencję zużyję częściowo na zaangażowanie asystenta i sił technicznych, częściowo na wydatki bieżące związane z tą pracą.

W związku z moim pobytem w Stanach Zjednoczonych zamierzam wprowadzić do kraju metodykę badań nad nowo-odkrytym czynnikiem Rh, nad indywidualizacją prątków duru brzusznego za pomocą bakteriofagów, nowe badania nad gruźlicą i rickettsiami i wiele innych. Metody te będziemy opracowywali w Państwowym Zakładzie Higieny. Ewentualne kredyty niezbędne dla tych badań będą zatym uwzględnione w budżecie P.Z.H. i zwrócę się o ne za pośrednictwem Naczelnego Dyrektora.

/Prof.Dr.Ludwik Hirszfeld/

FIGURE 1 | Prof. Ludwik Hirszfeld's letter to the Ministry of Health dated September 10, 1946 [in Polish]; source: Hirszfeld Institute's archive.



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Dr.med.Zdzisław Przybyłkiewicz
Kraków.ul.Czysta 18.

Kraków, dnia 22 kwietnia 1948r.

Jaśnie Wielmożny Panie Dziekanie !

Uprzejmie dziękuję za list z dnia 9.IV.b.r.ę i za wyrażenie zgody na pracę nad leczniczym zastosowaniem typowanych bakteriofagów.W tej sprawie zaszło jednak małe nieporozumienie.Obawiałbym się bowiem zacząć odrazu masową produkcję bakteriofagów z jednej strony, z drugiej zaś doświadczenie Zakładu Produkcji P.Z.H. w Krakowie uczy nas, że bardzo trudno jest w obecnym czasie uzyskać sprawozdania z wyników jakie lekarze wzgl.szpitale uzyskują stosując nasze preparaty.Dlatego też pracę swoją planowałem w ten sposób, aby można było na miejscu w Krakowie zorientować się co do wartości leczniczej typowanych bakteriofagów.W porozumieniu z prof.Kostrzewskim miałem zamiar wykorzystać do tego celu materiał kliniczny Oddziału Zakaźnego Szpitala św.Łazarza i przygotowywać bakteriofagi indywidualnie dla każdego poszczególnego chorego. W ten sposób pomyślana praca zazębiałaby się o badania nad typowaniem bakteriofagowym pałeczek durowych prowadzone przez Kol.Bileka w tut:Filii PZH.Masową produkcję możnaby ewentualnie podjąć po uzyskaniu pewnych własnych wyników.Proszę uprzejmie o wiadomość, czy w ten sposób zaplanowana praca odpowiadałaby Panu Dziekanowi.

Łączę wyrazy prawdziwego szacunku i poważania

Z. Przybyłkiewicz

Zdzisław Przybyłkiewicz.

FIGURE 2 | A letter of the director of the Medical Microbiology Institute at the Jagiellonian University in Kraków, Dr. Zdzisław Przybyłkiewicz to Prof. Ludwik Hirszfeld dated April 22, 1948 [in Polish]; source: Hirszfeld Institute's archive.

be recognized. Notably, Przybyłkiewicz established the Vaccine Production Plant in Kraków that initiated production of phage preparations and was later transformed into IBSS BIOMED S.A., the company which continues interest in phage production to this day (the company's history is available at https://www.biomed.pl/en/company/history/).

In the following correspondence with the Deputy Minister of Health, Jerzy Sztachelski, Hirszfeld stated that the methodology of phage typing of typhoid fever bacteria had spread to six branches of the PZH throughout Poland with the intention to include all branches by the end of 1949 (Meissner, 2012). What clearly emerges from these letters is a picture of a man who was truly devoted to his research and was able to effectively fight for funds during the rough postwar period.

On March 7, 1948, Hirszfeld gave a lecture at the meeting of the Wrocław Scientific Society entitled "*Walka świata niewidzialnego z pozawidzialnym*" [The battle of the invisible with the imperceptible] in which he fully emphasized the antibacterial potential of phage by saying "Phage act in such an unbelievable small quantity, they destroy bacteria so completely, their strength exceeds the body's immune capabilities" (Hirszfeld, 1948). One month later, in April 1948, he published an article in *Polski Tygodnik Lekarski* [Polish Medical Weekly] entitled "*Bakteriofagi i ich rola w rozpoznawaniu duru brzusznego*" [Phages and their role in the diagnosis of typhoid fever], where he continued his flattering opinion about phage and their specificity of action (Hirszfeld et al., 1948). In his book *Immunologia ogólna* [General Immunology] from 1949, he added, "The antibacterial activity of the immune system is much weaker than bacteriophages. The practical implications of this phenomenon should be further explored" (Hirszfeld, 1949). In a letter from 1950 to Prof. Feliks Przesmycki, Hirszfeld emphasized that phage research should be conducted at all Departments of Medical Microbiology in Poland (Meissner, 2012). We truly believe that Hirszfeld's words and actions deserve special attention as they took place at a time when phage research was almost completely abandoned in the countries west of Poland and no one paid serious attention to the potential of phage treatment. Hirszfeld was fully aware that his research must create a scientific basis for improving human health and that was the mission he was called to pursue (Bajer, 2003).

The Beginnings of the Hirszfeld Institute

As mentioned above, in December 1952 pursuant to Resolution No. 70 of the Polish Academy of Sciences, the Department of Medical Microbiology in Wrocław was transformed into the Institute of Immunology and Experimental Therapy. Naturally, Prof. Ludwik Hirszfeld became the first Director as his efforts led to the establishment of the Institute. He was not only the founder and first director. Hirszfeld shaped the scientific profile and activity of the Institute for the coming years. The Resolution of the Presidium of the Polish Government dated 1954 on the establishment of the Institute included plans for conducting research on lytic activity of phage. That Resolution was prepared mainly on the basis of Hirszfeld's previous work (Meissner, 2012). Officially, the Institute started its activity in February 1954, 1 month before Hirszfeld's death. The Institute was named after his founder, and these days it is internationally recognized under the name of the Hirszfeld Institute. Ludwik Hirszfeld was aware of the Institute's position and importance by describing it as the only one of its kind in Poland and significant among the world's medical and scientific institutions (Bajer, 2003). Several years later, Sulakvelidze et al. (2001) concluded that the most detailed studies published in English on the use of phages in clinical settings have come from the Hirszfeld Institute.

In the beginning, the Institute was situated in the old building of the Department of Medical Microbiology. An extremely important event in the history of the Institute was the construction of the new facilities located on 8 ha in the south of Wrocław. Its completion at the beginning of 1975 allowed all laboratories to be gathered in one place, which until then had been scattered all over the city. It also allowed an increase in and expansion of the scientific activity of the Institute. The new Institute's headquarters was a result of persistent and untiring efforts by Prof. Stefan Ślopek, the second director of the Institute (Duś and Ługowski, 1996).

Stefan Ślopek

After the death of Ludwik Hirszfeld, in September 1954, the aforementioned Prof. Ślopek, former Head of the Department of Clinical Microbiology of the Silesian School of Medicine, became the Director of the Institute (Duś and Ługowski, 1996). He served this role for 31 years remaining the longest-serving Director of the Hirszfeld Institute. During his directorship, Prof. Ślopek contributed greatly to the expansion of phage therapy in the form that was possible in his time. The Institute expanded his phage bank and produced phage preparations (phage lysates) directed against various pathogens. Those preparations were then distributed mainly among local hospitals and outpatient clinics that were supposed to give feedback on the results of treatment. The treatment in such a form involved more than 1000 patients. Prof. Ślopek's team published many articles, mainly in the local journals, reporting very high success rates (oscillating around 90%) for the results of phage therapy (Ślopek et al., 1983a,b, 1984, 1985a,b,c, 1987; Kucharewicz-Krukowska and Ślopek, 1987; Weber-Dąbrowska et al., 1987). Moreover, those reports have emphasized the safety of the therapy and very few side effects. Although today's analysis of phage therapy does not match those success rates, it should be kept in mind that most of the patients treated in the past were acute cases of bacterial infections that are more susceptible to therapy than chronic patients whose infections are much more difficult to control (Międzybrodzki et al., 2012). In addition, Ślopek's team did not personally monitor the patients but had to rely on other centers' reports with all the possible shortcomings of this type of "remote" evaluation.

Special attention should be given to the research on phage against *Shigella* species causing dysentery (*Shigella sonnei* and *Shigella flexneri*), which was extensively developed at Hirszfeld Institute at that time (Mulczyk and Kucharewicz-Krukowska, 1957; Metzger and Mulczyk, 1958; Ślopek et al., 1961, 1968a,b; Mulczyk et al., 1967; Krzywy, 1971; Mulczyk and Ślopek, 1971; Kostrzewski et al., 1974). Dysentery was a significant epidemiological problem in the 1960s and early '70s in Poland

and other eastern countries, especially among children. In 1971, the Reference Dysentery Center was established at the Hirszfeld Institute. The Center was responsible for collection from various European health centers of over 5,000 *Shigella* strains that were later tested for their phage sensitivity. This work resulted in the formation of an international set of phages for typing *Shigella* species and served as a basis to create polyvalent phage cocktails for prophylaxis of *S. sonnei* and *S. flexneri* infections. Such a phage cocktail was widely applied for epidemiological purposes to suppress dysentery outbreaks. Mulczyk and Ślopek reported the production of more than 1,000 L of polyvalent specific phage preparations by the Kraków Serum and Vaccine Laboratory for use in children's institutions in 1972 (Mulczyk and Ślopek, 1974). Notably, research on *Shigella* phage spread throughout other centers in Poland such as Gdańsk, Katowice, Łódź, Poznań, and Warsaw (Gawronowa et al., 1974; Kokocińska and Rokossowski, 1974; Nowak-Lipińska and Libich, 1974; Szkudlarek, 1974). Among phages deposited in collection of the Hirszfeld Institute, there are still few historic ampoules with *Shigella* phage cocktails from that period (**Figure 3**).

Further, Kańtoch and Mordarski published their preliminary and pioneer results on phage affinity to mouse cancer cells *in vitro*. The authors hypothesized that some of the cancer cells could contain active phage virions inside them (Kańtoch and Mordarski, 1958). One of the topics investigated at Hirszfeld Institute was an immunogenic effect of phage preparations on the induction of anti-phage antibodies in patients undergoing treatment (Kucharewicz-Krukowska and Ślopek, 1987). This area of research has been greatly expanded at the Hirszfeld Institute. In addition, Kańtoch investigated various aspects of phage phagocytosis (Kańtoch et al., 1958).

Noteworthy is also the series of articles published in the 1960s by Anna Przondo-Hessek from the Medical Academy in Wrocław (today: Wrocław Medical University) which covers the morphology, biology, and therapeutic applications of *Klebsiella* phage from her collection (Przondo-Hessek and Ślopek, 1967a,b; Przondo-Hessek et al., 1967).

Warsaw Phage Treatment

In the 1950s and 1960s, Wrocław was not the only phage center in Poland. Besides aforementioned research on phage against *Shigella* species, therapeutic connotations of phage were investigated by Dr. Michał Lityński whose detailed work dedicated to *E. coli* phages in the treatment of bacterial infections such as dysentery and renal pelvis inflammation was published in 1950 by Polish journal *Medical Review* [Przegląd Lekarski] (Lityński, 1950). Lityński focused on his experience gained during WWII and the findings of other authors. Similar to the work of Dr. Jerzy Jasieński, described above, Dr. Lityński's critical approach is noticeable. He emphasizes the necessity of close cooperation between bacteriologist and physician in order to achieve valuable data and increase the chances of a favorable outcome of treatment.

In the Medical Academy of Warsaw (today: Medical University of Warsaw) Witoszka and Strumiłło applied phages to surgical wounds infected by antibiotic-resistant *Staphylococcus* strains (Witoszka and Strumiłło, 1961). The authors were fully aware of increasing antibiotic-resistance, noting that nearly 100% of coagulase-positive staphylococcal strains were already resistant to penicillin and streptomycin. Phage preparations were provided by the Sanitary and Epidemiological Station in Warsaw and its Phage Unit run by a longtime phage specialist Irena Lipska about whom we wrote earlier. In the years 1960–1962, among 50 patients who underwent phage treatment, a good clinical outcome (including pathogen eradication) was obtained in 38 of them. Interestingly, phage preparations were applied in the form of an aerosol.

DEMOCRATIC TRANSITION IN POLAND

In 1989, Poland once again in the XX century had to face political and economic challenges. It was the first country from the so-called Eastern bloc to start a democratic revolution that eventually led to the fall of the Berlin Wall and the creation of a whole new Europe. Despite numerous obstacles, both financial and political, phage treatment at Hirszfeld Institute was not interrupted. The following years brought new articles focused on the promising clinical outcome of phage therapy in humans published both in Polish and in English (Weber-Dąbrowska et al., 1996, 1997, 2003). From the Institute's brochure published in 1996 we learn that the main research areas of Bacteriophage Laboratory were identification of bacterial strains from clinical specimens, selection of therapeutic phage and preparation of phage lysates, and their application in collaboration with clinics and hospitals (Duś and Ługowski, 1996). In 2000, a comprehensive review with an update on Hirszfeld Institute's experience in phage treatment was published (Weber-Dąbrowska et al., 2000). This article provides a synopsis of clinical outcome in 1307 patients aged from 4 weeks to 86 years old treated in the years 1987–1999.

In 1999, Prof. Andrzej Górski became the Institute's Director and later the Head of Bacteriophage Laboratory, a role he still serves. In the early 2000s, our team has formulated a new hypothesis on possible interactions between phages and the immune system including a protecting immunomodulatory role of gut phages (Górski et al., 2003; Górski and Weber-Dąbrowska, 2005). In addition, we postulated that phage can translocate from the gut and mediate their anti-inflammatory and immunomodulating activities in other organs and tissues (Górski et al., 2006). Those assumptions have been fully confirmed by the recent data of other authors (Barr et al., 2013a,b; Lehti et al., 2017; Nguyen et al., 2017; Barr, 2019). Moreover, we have further advanced our hypothesis by pointing out that various phages may mediate different immunomodulatory functions (Górski et al., 2019b).

Phage Therapy Unit

In 2005, 1 year after Poland's admission to the European Union, the PTU at the Hirszfeld Institute has started its activity as the first ethically approved phage treatment facility in Europe. Establishing the PTU was possible thanks to Prof. Andrzej Górski's efforts. This center continues the rich tradition of PT in Poland, which dates to the early

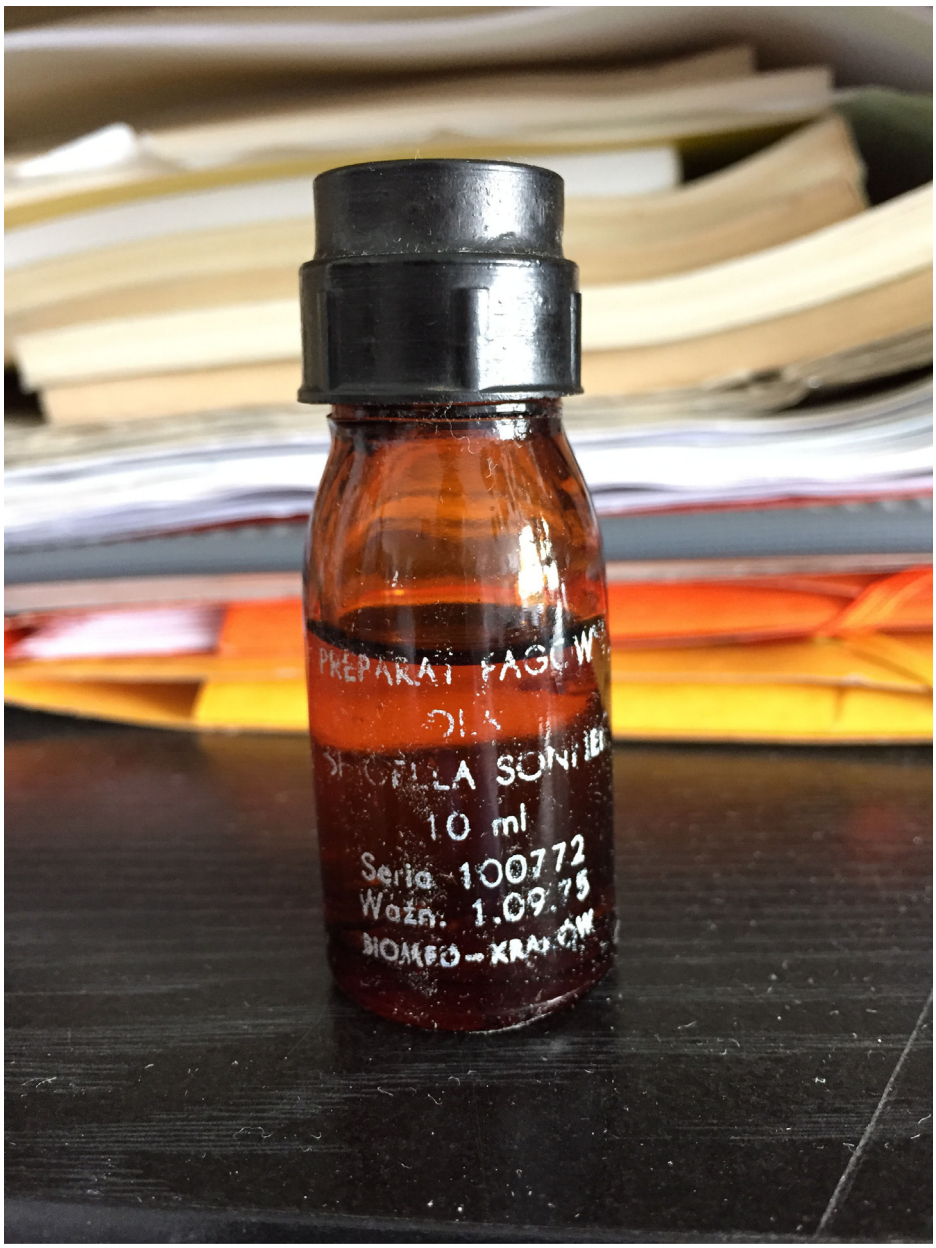

FIGURE 3 | A 10-mL ampoule with *Shigella sonnei* phage cocktail intended for oral administration. Phage cocktail was manufactured by BIOMED in Kraków (July 10, 1972) according to the instructions developed at the Hirszfeld Institute. Source: Private archive of Maciej Żaczek.

1920s (Międzybrodzki et al., 2012). PTU has paved the way for compassionate use of phage therapy in modern medicine and shaped the current state of knowledge concerning the experimental use of bacterial viruses. The Phage Therapy Unit is supported by the Bacteriophage Laboratory of the Hirszfeld Institute, which carries out phage typing procedures, prepares the phage formulations for patients, and performs some other tests within experimental phage therapy. Currently, the Bacteriophage Laboratory possesses one of the largest therapeutic phage collections, which consists of over 850 described and cataloged phages against the most common bacterial human pathogens. To date, over 700 patients have been subjected to phage treatment at PTU. The phage therapy is conducted on an outpatient basis under the protocol of an experimental program, "Experimental phage therapy of drug-resistant bacterial infections, including MRSA infections," approved by an Independent Bioethics Committee (opinion No. KB-349/2005). The program is supervised by Prof. Andrzej Górski, the head of the Phage Therapy Unit and the Bacteriophage Laboratory.

Phage scientists from the Hirszfeld Institute continue their publishing activity. In 2014, we published a book on phage therapy, *Phage Therapy: Current Research and Applications* [eds.

International Committee on Taxonomy of Viruses

VIROLOGY DIVISION – IUMS

28 May 2019

Beata Weber-Dąbrowska, PhD
Principal Specialist, Bacteriophage Laboratory
Ludwik Hirszfeld Institute of Immunology and Experimental Therapy
Polish Academy of Sciences
Wrocław
Poland

Dear Dr Weber-Dąbrowska,

This letter is to register the fact that a new genus has been named in your honour in the recently updated taxonomy of viruses. The original proposal (2018.007B) was made to the ICTV by 30 of your international colleagues and noted that the genus *Webervirus* was "named in honour of Beata Weber-Dąbrowska who has worked at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy for over 40 years on phage therapy". This genus is now featured in the official taxonomy at the ICTV website. I have reproduced the relevant portion below, expanded to show the names of the species in the genus.

On behalf of the ICTV, I offer congratulations for this recognition of your significant and sustained contribution to phage research.

− **Order:** *Caudovirales*	5 families
+ **Family:** *Ackermannviridae* Member of *Caudovirales*	2 subfamilies, 4 species
+ **Family:** *Herelleviridae* Member of *Caudovirales*	5 subfamilies, 3 species
+ **Family:** *Myoviridae* Member of *Caudovirales*	5 subfamilies, 68 genera, 3 species
+ **Family:** *Podoviridae* Member of *Caudovirales*	3 subfamilies, 31 genera, 7 species
− **Family:** *Siphoviridae* Member of *Caudovirales*	11 subfamilies, 176 genera, 1 species
+ **Subfamily:** *Arquatrovirinae* Member of *Siphoviridae*	3 genera, 1 species
+ **Subfamily:** *Bclasvirinae* Member of *Siphoviridae*	5 genera
+ **Subfamily:** *Chebruvirinae* Member of *Siphoviridae*	1 genus
+ **Subfamily:** *Dclasvirinae* Member of *Siphoviridae*	2 genera
+ **Subfamily:** *Guernseyvirinae* Member of *Siphoviridae*	3 genera
+ **Subfamily:** *Mccleskeyvirinae* Member of *Siphoviridae*	2 genera
+ **Subfamily:** *Mclasvirinae* Member of *Siphoviridae*	2 genera
+ **Subfamily:** *Nclasvirinae* Member of *Siphoviridae*	3 genera
+ **Subfamily:** *Nymbaxtervirinae* Member of *Siphoviridae*	2 genera
+ **Subfamily:** *Pclasvirinae* Member of *Siphoviridae*	3 genera
− **Subfamily:** *Tunavirinae* Member of *Siphoviridae*	8 genera, 1 species
+ **Genus:** *Eclunavirus* Member of *Tunavirinae*	1 species
+ **Genus:** *Hanrivervirus* Member of *Tunavirinae*	1 species
+ **Genus:** *Rogunavirus* Member of *Tunavirinae*	9 species
+ **Genus:** *Rtpvirus* Member of *Tunavirinae*	2 species
+ **Genus:** *Sertoctavirus* Member of *Tunavirinae*	1 species
+ **Genus:** *Tlsvirus* Member of *Tunavirinae*	3 species
+ **Genus:** *Tunavirus* Member of *Tunavirinae*	7 species
− **Genus:** *Webervirus* Member of *Tunavirinae*	6 species
Species: *Enterobacter virus F20* Member of *Webervirus*	
Species: *Klebsiella virus 1513* Member of *Webervirus*	
Species: *Klebsiella virus KLPN1* Member of *Webervirus*	
★ **Species:** *Klebsiella virus KP36* Member of *Webervirus*	
Species: *Klebsiella virus PKP126* Member of *Webervirus*	
Species: *Klebsiella virus Sushi* Member of *Webervirus*	

With best regards,

ICTV President

FIGURE 4 | A congratulatory letter of the President of the International Committee on Taxonomy of Viruses, Dr. Andrew J. Davison to Dr. Beata Weber-Dąbrowska dated May 28, 2019; source: private archive of Dr. B. Weber-Dąbrowska.

Borysowski et al. (2014)]. The book provides comprehensive coverage of the topic with a focus on current research and emerging applications of phages and provides evidence of the unique position of the Hirszfeld Institute in the scientific world. Eric Keen from Washington University of St. Louis and Sankar Adhya from the United States Department of Health and Human Services and National Institutes of Health in their review described this position as a valuable resource for anyone interested in phage biology and/or biomedical significance that presents a compelling case that phage-based medicine is an idea whose time has come (Keen and Adhya, 2015). Recently, the second book, entitled *Phage Therapy: A Practical Approach*, has also been published by Springer [eds. Górski et al. (2019a)]. This book gives a detailed insight into the current state of the art of the therapeutic application of bacteriophages in different conditions and is, therefore, a valuable resource for individuals engaged in the medical application of novel phage therapies.

To salute the 100th anniversary of the discovery of bacteriophages and the 10th anniversary of the establishment of the Phage Therapy Unit in Wrocław, the Hirszfeld Institute organized an international conference "Clinical Phage Therapy," held on September 26, 2015. In July 2018, the Hirszfeld Institute co-organized in Wrocław, Poland, the 5th edition of one of the biggest and most prestigious phage conferences (sponsored by the International Society for Viruses of Microbes) – Viruses of Microbes 2018 (conference leaflet available at: http://meetings.embo.org/event/18-virus-microbe). The main organizers were Prof. Krystyna Dąbrowska from our Bacteriophage Laboratory together with Prof. Zuzanna Drulis-Kawa from the University of Wrocław. This major meeting summarized the world's current research and trends in phage applications and brought together the most prominent scientists focusing on phage research.

In 2019, a longtime phage researcher, who has been working at the Hirszfeld Institute for nearly 50 years and devoted her entire career to phage, Dr. Beata Weber-Dąbrowska, has been honored in a very special way by the International Committee on Taxonomy of Viruses (ICTV). A new genus of bacterial viruses, *Webervirus*, has been named after her and is now featured in the official virus taxonomy at the ICTV website (**Figure 4**). Dr. Beata Weber-Dąbrowska has co-authored more than 100 articles in the phage field so far.

FINAL THOUGHTS

Undoubtedly, establishing the PTU at the Hirszfeld Institute was a milestone in an over 100-year phage treatment tradition in Poland. These days, the PTU is the key, internationally recognized, site dedicated to experimental phage treatment, which so far has admitted hundreds of patients from all over the world.

To date, phage scientists from the Hirszfeld Institute have published over 100 articles in peer-reviewed journals (including pioneer, widely cited publications on different aspects of phage therapy in humans), submitted numerous international patent applications covering phage isolation, preparation, and application, participated in several conferences dedicated to phage research and become editors of recognized scientific books in the phage field. Despite the long tradition and achievements, we have always been very careful in assessing the effectiveness of phage treatment recognizing the fact that only clinical trials performed in line with EBM can determine this effectiveness.

In light of those facts, it is clear that Polish phage therapy has a rich and productive history. Our Phage Therapy Center has continued in its best tradition, and, based on past achievements, it has advanced the therapy by expanding the relevant knowledge with the aid of planning decisive clinical trials. At the same time, we envisage novel non-bacterial potential application of phage therapy (currently referred to as "drug repurposing"). Undoubtedly, future perspectives for phage application look no less interesting than past achievements.

The undisputed progress and significance of phage therapy in Poland would not have been possible without the contributions of Polish scientists from other centers. They did not carry out clinical phage therapy themselves, yet their basic studies and experimental work in animals have contributed greatly to the current high position of Polish phage research and therapy in the international arena. One should mention Krystyna Dąbrowska from our laboratory, Zuzanna Drulis-Kawa (University of Wrocław), Małgorzata Łobocka (Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw), Jarosław Dastych (Institute of Medical Biology in Łódź), Grzegorz Węgrzyn (University of Gdańsk), and Romuald Gryko (Military Institute of Hygiene and Epidemiology in Puławy), among others. This list is certainly not exhaustive.

The survival and further development of phage research on Polish soil is the best example that science stands above political turbulences and economic obstacles, neither of which have been lacking over the past century in Poland.

AUTHOR CONTRIBUTIONS

MŻ wrote and revised the manuscript, reviewed available publications and documents. BW-D wrote part of the manuscript, provided unique documents regarding history of the Hirszfeld Institute along with comments and personal insight. RM and MŁ-S revised the manuscript. AG is an originator of the subject and wrote parts of the manuscript.

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REVIEW
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Phages in Therapy and Prophylaxis of American Foulbrood – Recent Implications From Practical Applications

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American foulbrood is one of the most serious and yet unsolved problems of beekeeping around the world, because it causes a disease leading to the weakening of the vitality of honey bee populations and huge economic losses both in agriculture and horticulture. The etiological agent of this dangerous disease is an extremely pathogenic spore-forming bacterium, *Paenibacillus larvae*, which makes treatment very difficult. What is more, the use of antibiotics in the European Union is forbidden due to restrictions related to the prevention of the presence of antibiotic residues in honey, as well as the global problem of spreading antibiotic resistance in case of bacterial strains. The only available solution is burning of entire bee colonies, which results in large economic losses. Therefore, bacteriophages and their lytic enzymes can be a real effective alternative in the treatment and prevention of this *Apis mellifera* disease. In this review, we summarize phage characteristics that make them a potentially useful tool in the fight against American foulbrood. In addition, we gathered data regarding phage application that have been described so far, and attempted to show practical implications and possible limitations of their usage.

Keywords: American foulbrood, *Paenibacillus larvae*, honey bee, bacteriophages, endolysin, antibiotic resistance

INTRODUCTION

The honey bee (*Apis mellifera*) is an important element of natural environment that play a vital role in the process of pollination, and contributes to the improvement and maintenance of flora biodiversity (Morse and Calderone, 2000). Bees' activity also provides such valuable products as honey, bee pollen, propolis, bee wax, and royal jelly, which are widely used by humans in various industries, including food and diet supplement production, cosmetology, natural medicine

and pharmacology. Unfortunately, a significant decrease in the number of bee colonies has been observed worldwide in the last few decades. Much attention has been given to colony collapse disorder (CCD), described as an abnormal phenomenon based on the disappearance of the majority of worker bees in a colony; only the queen, lots of food and a few nurse bees remain in the nest to care for the remaining immature bees (van Engelsdorp et al., 2009; van Engelsdorp and Meixner, 2010). In recent years, honey bees have been exposed in the environment to many adverse factors, which include the chemicalization of modern agriculture, large-scale use of plant protection products, environmental degradation, as well as diseases caused by different pathogens and parasites (Cox-Foster et al., 2007; Dainat et al., 2012). In reality, all of these factors tend to overlap and interact, which means that their synergistic action can cause health problems in bee colonies, such as the abrupt disappearance of worker bees from the colony.

American foulbrood (AFB), caused by *Paenibacillus larvae*, is one of the most infectious, dangerous, lethal and easily spreading diseases of *Apis mellifera* caused by different pathogens and parasites. Despite the name, AFB is classified as a notifiable disease with a worldwide distribution in almost all beekeeping regions in each of the five continents (Alippi and Aguilar, 1998). The causative pathogen has been described by White (1906) as *Bacillus larvae*, a Gram-positive, spore-forming bacterium (Genersch et al., 2006) that can produce even more than one billion spores per infected larva (Shimanuki, 1997; OIE (World Organization for Animal Health), 2018). Infectious spores are transferred within or between colonies by worker bees or by beekeeping practices (Sturtevant, 1932; Lindström et al., 2008a,b). Spores are extremely long-lived (they can survive even more than several decades in honey or on hive equipment (Hasemann, 1961; Genersch, 2010) and resistant to unfavorable conditions, e.g., heat and chemical agents, thus they are very hard to remove (Genersch, 2010). Unfortunately, conventional antimicrobial therapies are only effective for the vegetative forms of bacteria, and so far AFB has proved impossible to eradicate anywhere using all available and allowed methods of treatment and prevention. The use of antibiotics to treat AFB is not a permanent solution due to the production of resistant spores and increase in antibiotic resistance in bacterial cells (Lodesani and Costa, 2005; Alippi et al., 2007); they can also contaminate honey, which could be dangerous for humans consuming this product (Ortelli et al., 2004; Martel et al., 2006; Saridaki-Papakonstadinou et al., 2006; Meeraus et al., 2015; Muriano et al., 2015). This is the reason why antibiotic application in AFB treatment has been banned in most European countries (Genersch, 2010; Forsgren et al., 2018). In many countries, disease control even includes burning of infected colonies that generates huge economic losses. Problems associated with the control and treatment of infected colonies result in a significant decrease in honey bee populations, beekeeping industry and, in consequence, agricultural production all over the world.

Bacteriophages may be a promising solution in the treatment and prevention of AFB spread in honey bees (Tsourkas, 2020). These bacterial viruses are commonly found in the biosphere (Clokie et al., 2011). A recent study has demonstrated that phages (both lytic and temperate) may be part of the honey bee gut microbial community (Bonilla-Rosso et al., 2020), participating in its structure modulation, which affects honey bee health (Deboutte et al., 2020). Phages are natural structures, safe and well-tolerated by higher organisms, including humans, and can also be safe for bees. Phages exhibiting lytic activity cause destruction and decay only of their bacterial host, without disturbing the composition of the natural gut microflora (Cieplak et al., 2018), and thus they undoubtedly can be applied as therapeutics. Phage ability to amplify at the site of infection is their another advantageous feature, which is why they are called "self-dosing." Furthermore, it has been suggested that phages may be used in the food industry, preventing the spread of pathogenic bacteria, degradation of food products and also promoting safe environment in animal and plant food production (Sillankorva et al., 2012). Isolation of new therapeutic phages for these purposes is a relatively simple, inexpensive, and rapid process. The use of phages in prophylaxis and treatment of bacterial diseases is a targeted method, with high specificity for the host of antimicrobial activity, less expensive and safer than conventional antibiotic treatments (Fernández et al., 2019). What is more, US Food and Drug Administration (FDA) approved a phage preparation as food additives in 2006, with a status of generally recognized as safe (GRAS) (García et al., 2008; Moye et al., 2018).

Knowledge of the potential use of bacteriophages in the fight against AFB is sparse, and a small percentage of studies devoted to this subject contribute to this situation. Therefore, in this article, we present data concerning phage application against *P. larvae* infections.

AMERICAN FOULBROOD

Pathogenesis and Epidemiology

Endospores of *P. larvae* are the only direct etiological factor of AFB, whereas, vegetative forms can also be harmful to bees through toxin production (Mahdi and Fisher, 2018). The species *P. larvae* comprises four different genotypes – named ERIC I-IV – based on enterobacterial repetitive intergenic consensus (ERIC) primers (Genersch and Otten, 2003; Genersch et al., 2006) that modulate infection with varying degree of pathogenicity. These types have different phenotypic characteristics, including colony and spore morphology, metabolic capacity, sporulation and virulence level (Neuendorf et al., 2004; Genersch et al., 2005, 2006; Forsgren et al., 2008; Rauch et al., 2009; Saville, 2011; Poppinga et al., 2012). A new ERIC genotype has been recently discovered – *Paenibacillus larvae* ERIC V (Beims et al., 2020). A comparison of the virulence of genotypes is presented in **Table 1**. Epidemiological studies showed that ERIC I and II are the most frequently isolated genotypes from infected colonies (Alippi et al., 2004; Peters et al., 2006; Antúnez et al., 2009; Loncaric et al., 2009), and these strains usually cause AFB epidemics (Fünfhaus et al., 2018). Each of these genotypes causes specific differences in *P. larvae* virulence, corresponding to the time of killing infected larvae (Genersch et al., 2005, 2006). *P. larvae* with genotype ERIC II are faster, with LT100 (lethal time

TABLE 1 | *P. larvae* genotypes and their characteristics (Genersch, 2010; Beims et al., 2020).

Genotype	ERIC I	ERIC II	ERIC III	ERIC IV	ERIC V
Species	*P. larvae*		*Paenibacillus larvae subsp. pulvifaciens*		*P. larvae*
Virulence	Kills larvae within 12 days	Shows the highest lethality	Kills larvae within 7 days		Kills even after 3 days
Frequency	Most frequent genotype, found throughout the world	Isolated worldwide, especially in Europe	Not identified in recent decades		Identified in Spain

to 100% population mortality) of approximately 7 days, when compared to members of genotype ERIC I that causes slower larva death (LT100 of approximately 12 days) (Genersch, 2007).

American foulbrood only affects the initial stages of bee development. Bees exhibit hygienic behavior which includes innate and hereditary behaviors associated, for example, with effective removal of sick/damaged brood by bees to prevent the emergence, spread and transmission of diseases of adult bees and brood. Certain studies demonstrated that some bees, which presented higher hygienic behavior, could better control brood disease, including AFB infection in colony conditions (Palacio et al., 2000; Spivak and Reuter, 2001). Chen et al. (2000) showed that a dose of spores used to inoculate *A. mellifera* and *A. cerana* larvae of the same age would cause 95% mortality in the former case and only 47.1% mortality in the latter. As regards *A. cerana*, lower levels of infection were caused by the removal of 82.2% inoculated larvae by adult bees before reaching the capped stage. However, it is difficult for bees to completely overcome the infection caused by *P. larvae* due to the high infectivity of *P. larvae*, the ability of bacteria to produce spores, as well as the spreading pathway, proliferation and the fact that no symptoms are noticed at the initial stage of infection.

American foulbrood usually spreads horizontally, but can also spread vertically, when colonies swarm (Fries et al., 2006). Horizontal transmission is observed when spores are distributed by adult bees within and between colonies, facilitating spreading of the disease to healthy larvae and colonies (Fries et al., 2006; Poppinga and Genersch, 2015). Another way of AFB spreading is by robber bees, which prey on colonies weakened by AFB infection and may take contaminated honey back to their hives and, as a consequence, spread the disease to other colonies and apiaries. Beekeepers can also be a vector through unintentionally using the same equipment for sick and healthy colonies (Lindström et al., 2008a,b; Pentikäinen et al., 2008). Vertical transmission is observed in honey bees during reproductive swarming (Fries and Camazine, 2001; Fries et al., 2006).

Honey bee larvae become infected during feeding with food contaminated with spores by adults nestmates (Yue et al., 2008). The susceptibility of larvae to disease caused by *Paenibacillus larvae* decreases with increasing age. Larvae until 12–36 h after hatching are most vulnerable to infection. During this time to successfully initiate infection larvae needs to consume a dose of 10 or less spores (Woodrow, 1942). Hansen and Brødsgaard (1999) showed that the mean infective dose needed to initiate infection in 24–28 h-old bee larvae is 8.49 $\pm$ 1.49 spores. Larvae older than 48 h become more resistant to infection so that no significant correlation of dose and mortality was observed after this time. The relationship between dosage and mortality is highly dependent on larval age, genetic constitution and bacterial strain (Genersch et al., 2005).

Once the spores reach the gut of a larva, they germinate and the vegetative forms of bacteria move into the gut tissues, where they multiply. After intestinal epithelial damage and invasion of the hemocoel infected larvae die after their cells are sealed and millions of infectious spores form in their remains. The AFB infection cycle is presented in **Figure 1**. Dried larval remains adhere to the cell walls and cannot be easily removed by bees, and thus the comb remains contaminated and is a source of spores that can spread within and between colonies. The lifecycle of *P. larvae* in honey bee can be divided into two stages. The first one is the time when spores germinate in the larval midgut, where the vegetative bacterial cells massively proliferate for several days without destroying the epithelial integrity and live on food ingested by the host (Yue et al., 2008). During this period, *P. larvae* metabolize different sugars, which are compulsory to support vegetative growth, by enzymes of the Embden-Meyerhof-Parnas, pentose phosphate, and Entner-Doudoroff pathways involved in carbohydrate metabolism (Julian and Bulla, 1971; Neuendorf et al., 2004; Djukic et al., 2014). During the second stage, the midgut epithelium is penetrated and the hemocoel is attacked by bacterial vegetative cells, which is synonymous of death of the larvae and destruction of larval remains (Neuendorf et al., 2004; Yue et al., 2008). When nutrients become scarce, the *P. larvae* population undergoes sporulation and the remains become brown and mucilaginous, which is the most characteristic clinical symptom of AFB (Lindström et al., 2008b; Poppinga and Genersch, 2015), known as the rope, because viscous larval remains form a ropy thread when drawn out with a match. This glue-colloid dries down and adheres to the cell wall forming a kind of hard scale consisting of billions of spores, and are highly infectious (Bailey and Ball, 1991; Gregorc and Bowen, 1998). According to Stephan et al. (2020), there is a relationship between spore count and disease and colony development.

Detection and Treatment of Infected Honey Bees

American foulbrood in many countries is a notifiable disease and is required by law to be reported to relevant government authorities. AFB diagnosis is based on the identification of the etiological agent and the presence of clinical symptoms. Symptoms of AFB disease can be detected during inspection of honey bee colonies. In a healthy colony, the comb cells have a compact structure with brood typical of bees at various developmental stages. While AFB is progressing, the brood

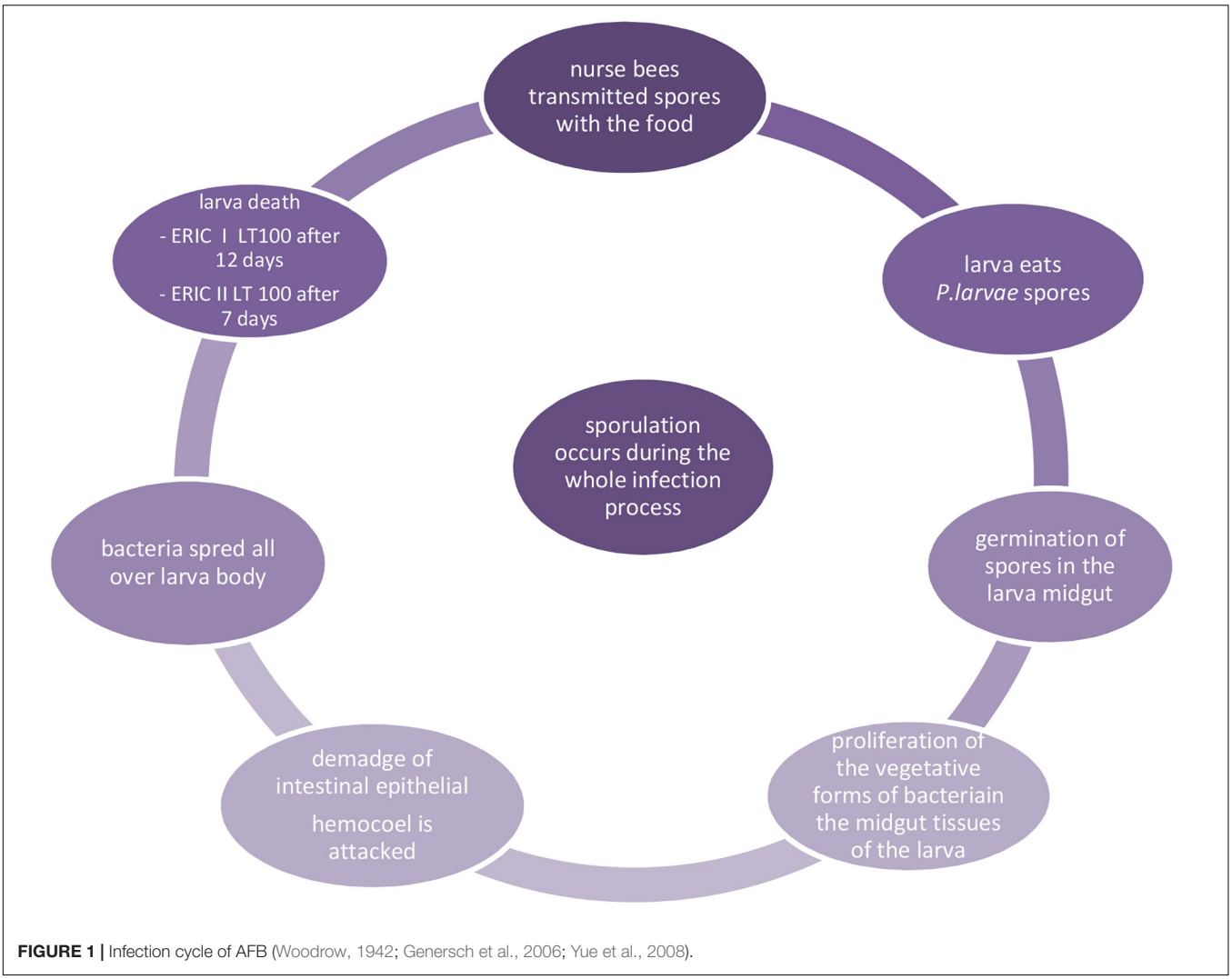

FIGURE 1 | Infection cycle of AFB (Woodrow, 1942; Genersch et al., 2006; Yue et al., 2008).

structure takes on an irregular appearance due to the presence of dead larvae or pupae in cells. AFB-infected combs are drier, darker, and have a slight foul odor. The cells in the comb have sunken caps (Shimanuki and Knox, 2000; Gliński et al., 2006). Choice of samples for testing depends on whether it concerns a suspicious or diseased honey bee colony or analysis in the context of an AFB monitoring or prevention program. Studies using alternative diagnostic methods showed that analysis of honey and bee samples collected at the entrance to the hive are of limited value, because not all samples (only 86 and 83%, respectively) collected from colonies presenting symptoms of infection were positive. Analysis of bee samples from the space of the brood nest, edge frame or honey chamber is more reliable (Gillard et al., 2008).

Microbiological characteristics, polymerase chain reaction (PCR), biochemical profiling, antibody-based techniques and microscopic identification techniques are most commonly used for *P. larvae* identification. Other methods that may also be used to identify this pathogen are based on testing bacteriophage sensitivity, immunological technique or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Stahly et al., 1999; Schäfer et al., 2014). It is also possible to detect *P. larvae* using microbiome analysis (Erban et al., 2017). Real-time PCR analysis of the 16S rDNA gene of *Paenibacillus* represents an alternative, rapid diagnostic tool (Chagas et al., 2010; Martínez et al., 2010; Alippi et al., 2014). Innovative methods, e.g., identification of endolysin cell binding domain (CBD), which targets *P. larvae*, may be suggested to identify bacterial strains that are the causative agent of AFB (Santos et al., 2019). The World Organization for Animal Health (OIE) has presented a broad outline of various diagnostic methods, but due to differences in sensitivity, the most appropriate of the described methods should be selected (OIE (World Organization for Animal Health), 2018). In addition, there are several selective media for *P. larvae* culture: *Paenibacillus larvae* agar (PLA), (Schuch et al., 2001), MYPGP agar (Dingman and Stahly, 1983), BHIT agar (brain–heart infusion medium supplemented with thiamine) (Gochnauer, 1973), J-agar (Gordon et al., 1973) and CSA (Columbia sheep-blood agar) (Hornitzky and Karlovskis, 1989).

The management of AFB spread reduction relies on different methods: the use of antibiotics, natural products or destruction of infected hives (Genersch, 2010). When the presence of bacteria and first clinical symptoms of AFB are detected, double resettlement can also be applied, but it is effective only when the disease is at an early stage of development (Ritter, 2012). Munawar et al. (2010) investigated the shook swarm method that could also be used for AFB control. The results showed significantly decreased spore load in bee mouths by starving them and shifting them to new, clean hives with new foundation sheets.

Nevertheless, burning colonies that exhibit AFB symptoms is considered the most effective control method to prevent spreading the disease and is usually a legal requirement. These restrictions particularly apply in EU and burning is recommended as the only way to destroy infected colonies (Genersch, 2010; Alippi et al., 2014). However, antibiotics are accepted for prophylaxis and treatment in the United States and Canada (Evans, 2003; Genersch, 2010). This forces the development of alternative, natural strategies for the prevention and control of AFB. Therefore, studies have been published that suggest application of essential oils (Fuselli et al., 2008; Chirila et al., 2011; Maggi et al., 2011; Gende et al., 2014; Kuzyšinová et al., 2014; Santos et al., 2014; Ansari et al., 2015), plant extracts (González and Marioli, 2010; Damiani et al., 2014; Hernández-López et al., 2014; Anjum et al., 2015; Piana et al., 2015), propolis (Antúnez et al., 2008; Bastos et al., 2008; Mihai et al., 2012; Bíliková et al., 2013; Wilson et al., 2015) or probiotics (Alonso-Salces et al., 2017; Daisley et al., 2020). In addition, *in vitro* studies showed antimicrobial activity of royal jelly from different geographical origins against *P. larvae* (Bachanová et al., 2002; Bíliková et al., 2009). Rumanovská et al. (2011) observed some potential for omega 3 polyunsaturated fatty acids in reducing the number of *P. larvae*. Another study showed that *Bacillus subtilis* isolated from honey bee guts and honey samples was able to inhibit *P. larvae* development. Alippi and Reynaldi (2006) detected in their research that other bacteria, e.g., aerobic spore-forming *Bacillus megaterium*, *Bacillus licheniformis* and isolates of *Bacillus cereus* also showed antagonistic effects on *P. larvae*. It was also observed that lactic acid bacteria, such as *Lactobacillus kunkeei* decreased the mortality of the brood infected with *P. larvae* (Arredondo et al., 2017). Unfortunately, the methods listed above target active infection, similarly to antibiotics, but do not destroy *P. larvae* spores.

ANTIBIOTIC RESISTANCE OF *P. larvae* STRAINS

Antibiotics are not fully effective antimicrobials when applied in AFB treatment, they can cause many deleterious effects, and do not destroy *P. larvae* spores; they treat symptoms, but do not cure the disease, because they prevent the multiplication of only the vegetative forms of bacteria (Genersch, 2010). Application of antibiotics may result in an imbalance in enteric homeostasis, e.g., a disturbance in the influence of honey bee gut microbiota on bee metabolism or immune response, and increase the chances of fungal infection (Raymann et al., 2017). Moreover, when used for a long time, they may cause selection of resistant mutants among different *P. larvae* strains (Tian et al., 2012; Alippi et al., 2014), leading to antibiotic ineffectiveness. Resistance genes are encoded by mobile genetic elements; bacterial strains acquire them as a result of horizontal gene transfer through phage transduction (Gómez-Gómez et al., 2019). There are data suggesting that antibiotic resistance genes can remain in the environment even for 30,000 years (D'Costa et al., 2011). Interestingly, these genes can be detected in food and transferred to different ecological niches (Godziszewska et al., 2016). Antibiotic residues were detected in different honey bee products, e.g., honey, wax or royal jelly (Hammel et al., 2008; Lopez et al., 2008; Bargańska et al., 2011), thereby reducing honey quality, and potentially affecting the vitality and longevity of bees. A serious threat to humans may be associated with the possibility of antibiotic residue accumulation in commonly consumed bee products (Ortelli et al., 2004; Saridaki-Papakonstadinou et al., 2006).

The World Health Organization (WHO) indicated antibiotic resistance as one of ten biggest threats to global health (World Health Organization (WHO), 2019). Antibiotic application causes the possibility of resistance development in *P. larvae*, and it has already been detected both in the United States, Canada, and Argentina isolates (Miyagi et al., 2000; Evans, 2003; Alippi et al., 2007). It may be acquired, e.g., via genetic transfer (by mobile genetic elements, e.g., plasmids) even between different bacterial genera from soil, and plasmids encoding antibiotic resistance genes were detected, e.g., in commercial honey (Alippi et al., 2014). Wild strains of *P. larvae* were proved to carry oxytetracycline resistance genes (Alippi et al., 2007). It has been demonstrated that resistance of wild *P. larvae* strains may reach even 58% of the samples (Alippi et al., 1999; Miyagi et al., 2000; Spivak, 2000; Murray and Aronstein, 2006; Mitrano et al., 2009). Elzen et al. (2002) reported that macrolide Tylosin Tartrate was more effective in controlling oxytetracycline-resistant *P. larvae*, with no effect on adult and larval bee mortality.

In the United States, antibiotics are permitted in the elimination of *P. larvae*. For decades, oxytetracycline was the only approved antibiotic used for this purpose. But since 2005, the FDA has approved four new products to control the disease. For example, the second antibiotic, tylosin tartrate (TYLAN, TYLOVET, TYLOMED-WS), was approved in 2005, whereas the newest antibiotic against AFB – lincomycin hydrochloride (LINCOMIX) – was approved in 2012 (FDA (Food and Drug Administration), 2020).

In the past, antibiotics and sulfonamides were used in EU in the treatment of colonic diseases. Current legislation (Regulation EEC2377/90 and amendments) prohibits the use of antibiotics and does not allow the presence of their residues in honey and hive-derived products, which prevents their application and, in consequence, limits the range of available methods to fight AFB.

P. larvae BACTERIOPHAGES AND THEIR CHARACTERISTICS

Because an increase is observed in the frequency of antibiotic resistance in bacteria, and stringent regulations prohibiting the

use of antibiotics in bee disease treatment, phages are suggested as components that may be intended to combat microbial resistance (NIH, 2014) as effective and safe agents in AFB treatment and prevention. Bacteriophages are naturally occurring bacterial viruses that can be found in the hive and honey bee organisms. Phages active against *Bacillus larvae* were isolated for the first time by Smirnova (1953) from bee larvae suffering from AFB. The source of phages can be: lysogenized bacteria (Gochnauer, 1955; Dingman et al., 1984), water, soil from the hive area (Popova et al., 1976; Valerianov et al., 1976; Ribeiro et al., 2019b), swabs from hive surfaces, beehive materials (Beims et al., 2015) wax, brood (Oliveira et al., 2015), larvae, adult workers and even cosmetics, e.g., containing honey as an ingredient (Merrill et al., 2014; Stamereilers et al., 2016; Yost et al., 2016, 2018; Walker et al., 2018; Tsourkas, 2020). They may be present in the material in which the host bacteria were isolated. **Figure 2** presents possible sources of *P. larvae* phage isolation. The phages showed high specificity for *P. larvae* and both lytic (Yost et al., 2016) as well as temperate phages were isolated (Dingman et al., 1984). According to some of the recent data, all so far described phages in *P. larvae* are lytic *in vitro* (Stamereilers et al., 2018), including those induced from prophages, while other study has indicated that they are all temperate (Tsourkas, 2020). Therefore, this classification may cause incompatibilities, and researchers should be very cautious, for example because phages firstly identified as lytic may prove to be induced from bacteria (i.e., they carry integrase genes in their genomes) after detailed analysis, including BLAST (Stamereilers et al., 2018). Strictly lytic phages are safe when applied in phage therapy, because they do not have the possibility to incorporate into the bacterial genome and transduce bacterial genes when compared to temperate ones (Górski et al., 2020). Temperate phages, especially those capable of transferring antibiotic resistance genes, are not safe and should be excluded from phage therapies. However, there are data suggesting that temperate phages could potentially find use in therapy (Chung et al., 2012; Meader et al., 2013), especially in the fight against AFB, as presented by Ghorbani-Nezami et al. (2015). Of course, for safety reasons, their application should be carefully considered. For example, inability to transduce should be proved at the gene level, as in the study of Ribeiro et al. (2019b) on vB_PlaP_API480, and only then phage application potential can be assessed and confirmed *in vivo*.

Morphologically, *P. larvae* phages were mostly identified as an elongated-capsid siphovirus and round-capsid siphovirus (Merrill et al., 2014), but myoviruses were also found (named Abouo, Davies, Emery, Jimmer1, and Jimmer2). The first group of siphoviruses contains phages Diane, Fern, Hayley (Stamereilers et al., 2016), BLA, PBL1, and PPL1c (Merrill et al., 2014). These phages have long, non-contractile tails and elongated capsids. The second siphovirus group contains only PBL3, which have a round capsid (Campana et al., 1991; Merrill et al., 2014). The size of siphovirus phages is approximately 300 nm; phage capsid is approximately 100 nm-long and

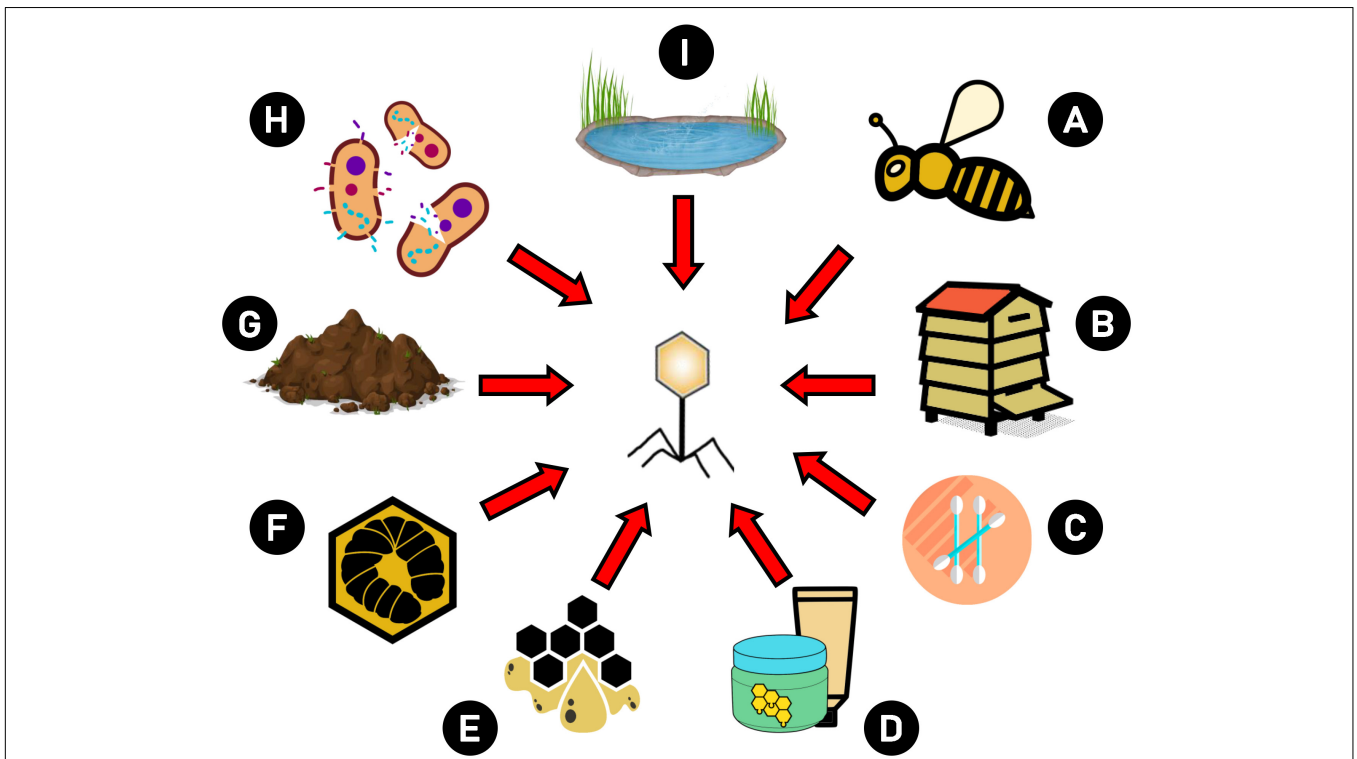

FIGURE 2 | Possible sources of *P. larvae* phage isolation. Phages intended to use against AFB may be isolated from different sources: **(A)** bees and bee products, **(B)** hive elements (e.g., combs), **(C)** swabs from hive area, **(D)** cosmetics which contain bee products, **(E)** wax, **(F)** larvae, **(G)** soil, **(H)** lysogenic bacteria, **(I)** water.

50 nm-wide, and tails are approximately 150–200 nm in length (Stamereilers et al., 2016). *Myoviruses* such as Abouo, Davies, Emery, Jimmer1, and Jimmer2 are similar in size to siphoviruses, with an average capsid height of 67.2 ± 3.2 nm and an average width of 64.1 ± 2.6 nm. The average tail length is about 122.0 ± 27.3 nm (Merrill et al., 2014). Ribeiro et al. (2019b) reported isolation of a *Podoviridae* phage, vB_PlaP_API480, with activity against *P. larvae*, characterized using transmission electron microscopy as a phage with an icosahedral capsid and a short 12 × 8 nm non-contractile tail, 58 nm in diameter.

Genetic Characteristics of Sequenced *Paenibacillus larvae* Bacteriophages

Phages specific to *P. larvae* were already identified in 1950, but genome sequencing was not possible at the time (Smirnova, 1953; Gochnauer, 1955). Phage phiIBB_Pl23, isolated in Portugal by Oliveira et al. (2013), was the first bacteriophage whose genome was fully sequenced. The following five phages: Abouo, Davies, Emery, Jimmer1, and Jimmer2 were isolated and sequenced in Utah, United States also in 2013 (Sheflo et al., 2013). They were first identified as phages specific to *Bacillus larvae*, however, after reclassification of the host bacterium (Genersch et al., 2006; Merrill et al., 2014), the names of phages were changed as specific to *Paenibacillus larvae*. Some publications considered these phages as *Paenibacillus* (Merrill et al., 2014), while other works did not classify them to this group of phages (Stamereilers et al., 2016, 2018; Tsourkas, 2020).

A new phage PG1 was identified in 2013, whose sequence was submitted to GenBank, but not published in any journal. After 2 years, phages Diva, Lily, Rani, Redbud, Shelly, Sitara (Carson et al., 2015), and Tripp (Abraham et al., 2016), isolated in North Carolina, were also sequenced. HB10c2 was isolated and sequenced in the same year in Germany (Beims et al., 2015). The next nine phages Diane, Fern, Harrison, Hayley, Paisley, Vadim, Vegas, Willow, and Xenia were isolated and described by scientist from the University of Nevada and Texas (Tsourkas et al., 2015). A thorough genomic sequence analysis of these phages was also performed. Phages were compared with each other and with other sequenced *P. larvae* phages; scientists additionally attempted to identify putative protein functions (Stamereilers et al., 2016). In 2014–2016, a large number of *Paenibacillus* phages was isolated by students from the Phage Hunters course at the Brigham Young University (BYU) (Merrill et al., 2018). In addition, the genomes of four *Paenibacillus* phages were sequenced in the Brigham Young University in 2018 (Yost et al., 2018). Stamereilers et al. (2018) analyzed and classified the genomes of *P. larvae* phages whose sequences were available in GenBank. In our opinion, the group of *P. larvae* phages includes many more sequenced phages and is still growing. Sequences available in GenBank are described as *Paenibacillus* phages, but some also as *Brevibacillus* phages, e.g., Abouo, Davies, Emery, Jimmer1 and Jimmer2; there are also phages without a group name, such as bacteriophage Lily, Sitara, Redbud, Shelly, Rani or Diva.

The genome size of *P. larvae* phages ranges from 35 kb (phage HB10c2) to 58 kb (phage Emery). Most *P. larvae* phages reproduce via lytic cycle on used bacterial strains *in vitro*, including those originally induced from prophages, such as Diane, PBL1c, and Xenia (Stamereilers et al., 2018). *Myoviridae* phages like Abouo, Dives, Jimmer1 and Jimmer2 are very similar to each other. For example, Jimmer1 and Jimmer2 share 99.8% average nucleotide identity. Abouo and Daives share 94.9% identity, but Emery differs from other phages (Merrill et al., 2014). Stamereilers et al. (2016) compared nine phages: Diane, Fern, Harrison, Hayley, Paisley, Vidim, Vegas, Willow and Xenia and found that phages Diane, Vadim, Vegas, and Hayley were highly similar to each other; the second similar pair was Harrison and Paisley, and the third: Fern and Willow. Xenia did not seem similar to any other examined phages, but it was found to be similar to phage Shelly isolated by another research group (Stamereilers et al., 2016). Furthermore, scientists classified 17 sequenced *P. larvae* phages into clusters and subclusters based on nucleotide sequence identity; *P. larvae* phages were classified into two main clusters: A and B. Cluster A contained phages Diane, Vadim, Vegas, Hayley, Harrison, Paisley, whereas phages Fern, Willow, Xenia, Diva, Rani, Redbud, Shelly, Sitara, HB10c2 and phiIBB_Pl23 were classified to cluster B. Phage Lily was very divergent from all other *P. larvae* phages and did not belong to any cluster. Cluster B was much more diverse and contained three subclusters and singletons. Similarity between clusters was low, approximately 40%. Differences between clusters included joining protein (gp9), prohead protease (gp10) and partly large proteinase protein. Cluster A phages had identical tail tape measure protein, which was different than the tail tape measure protein of Fern, Willow and Xenia from cluster B (Stamereilers et al., 2016).

After 2 years, the same research group compared 48 sequenced phages against *Paenibacillus larvae* (Stamereilers et al., 2018). They proposed a broader division into five clusters representing the following groups: Fern, Harisson, Vegas, Lily and Halcyone. The Fern cluster was the largest and contained 30 of 48 *P. larvae* phage genomes: Pagassa, Honeybear, Toothless, Tadhana, Fern, Willow, Lucielle, Saudage, BN12, Kawika, Kiel007, Redbud, Rani, Eltigre, HB10c2, Arcticfreeze, DevRi, Bloom, Jacopo, Likha, phiIBB_Pl23, Yerffej, Sitara, Diva, Shelly, Xenia, Leyra, PBL1c, Genki and Gryphonian. The second Halcyone cluster contained eight phages (Ash, Ley, C7Cdelta, Halcyone, Heath, Tripp, Scottie, and Unity), the Vegas cluster included seven phages (Diane, Vadim, Vegas Hayley, Dragolir, LincolnB, Wanderer), while the Harrison cluster contained only two phages: Harrison and Paisley, while phage Lily was a singleton. Phages in the Halcyone cluster were very distant from all other phages. To date, 56 *Paenibacillus* phages have been annotated in GenBank. **Supplementary Table S1**, which includes characteristics of all phages classified into this group. **Figure 3** presents a schematic picture that divides phages into individual groups, including the families to which they belong.

Approximately 90% of *P. larvae* phages show some similarities to other phage sequences from GenBank, about 50% of phage gene products have at least one sequence similarity match to proteins with putative function. The number of genes in

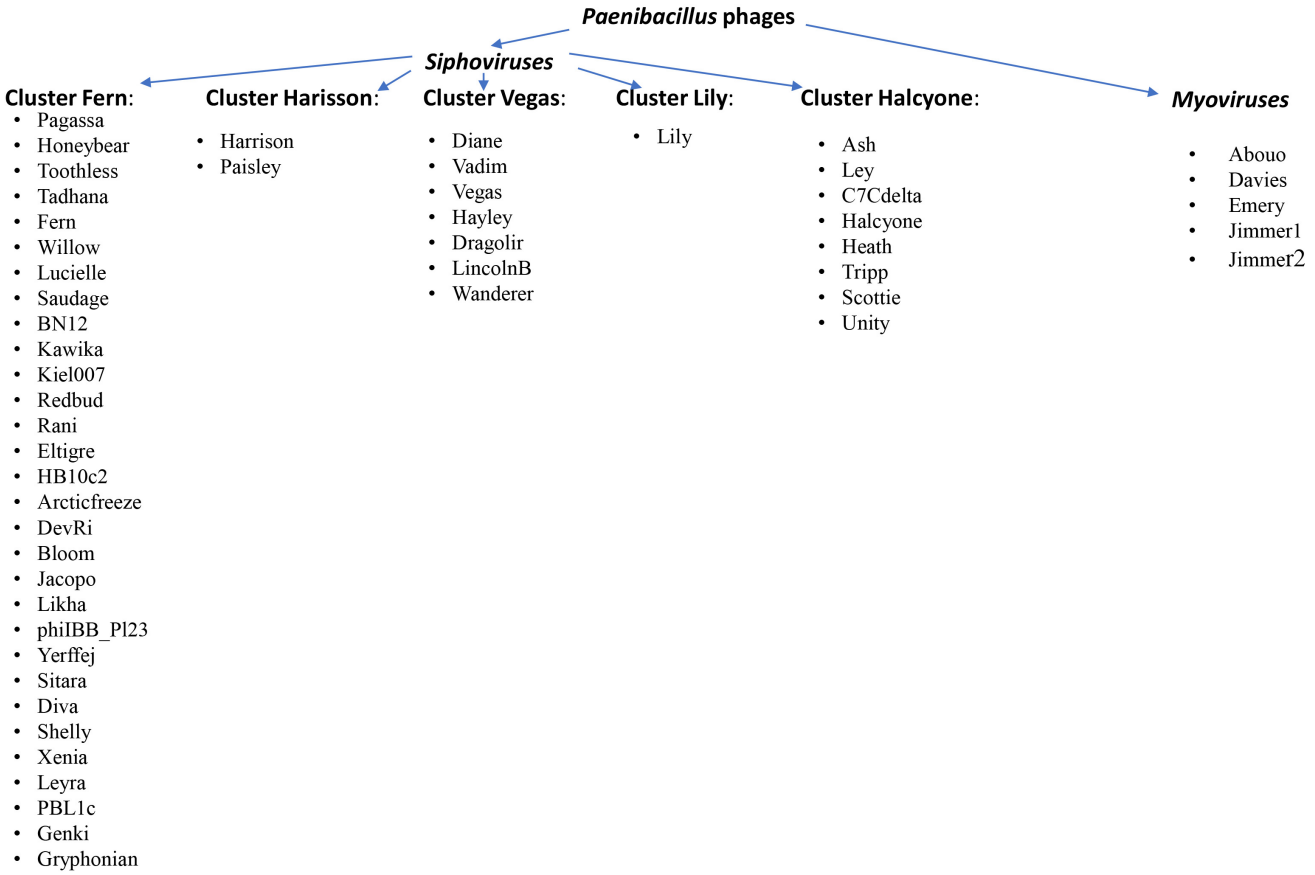

FIGURE 3 | Division of *Paenibacillus* phages into individual groups, including families to which they belong (Stamereilers et al., 2018; Tsourkas, 2020).

the *P. larvae* siphovirus genome ranges from 58 (HB10c2) to 91 (Scottie), with the number of genes changing linearly with genome size (Stamereilers et al., 2018). For myovirus, this number reaches even 102 genes (phages Emery, Jimmer1, and Jimmer2). These similarities enabled the determination of probable protein functions and classification into categories such as structural, assembly, lysis, regulatory, DNA replication and host-related function (Tsourkas, 2020). All *Myoviridae* and *Siphoviridae* phages have a conserved region, located at the start of the genome. This region is located around gp1 to gp17 and codes for virion structural proteins (Stamereilers et al., 2016). Gene products, such as small and large terminase, portal protein, protease and major capsid protein were identified in all phages. Major capsid proteins are encoded by gp5, gp7 or gp8, depending on the phage. This protein builds the phage capsid to the greatest extent. Head-tail joining protein is located at positions gp9 gp10 or gp11. Large terminase (gp2) is involved in DNA packing into empty capsids. Portal protein is involved in the DNA packaging process. Similarity between the architecture of portal oligomers and DNA packaging strategies suggests that portal protein plays the same role in a large number of viruses (Isidro et al., 2004). DNA replication, regulatory and host-related genes are located in the middle of the sequence and usually are not conserved (Tsourkas, 2020). These genes are the most diverse group of genes in phages and they differ significantly between individual phages.

All siphovirus phages encode host lysis genes, usually at position gp21, which codes for a conserved bacteriocin, a toxin produced by prokaryotes inhibiting the growth of competing bacteria (Stamereilers et al., 2016). This gene also has strong BLAST matches to unconfirmed holin-like protein (bhlA protein). *Myoviridae* phages also have holin-like protein but approximately at position gp34-36 (Merrill et al., 2014). DNA regulatory genes form the largest and most diverse group of genes. It consists of genes encoding endonucleases, transposases, integrases, methyltransferases and others. Tail proteins are coded by genes at position gp15-gp16 in all phages. These proteins could have catalytic activity that would allow the phages to enter the host.

PHAGE APPLICATION POTENTIAL

Since the first isolation of phages active against *P. larvae*, many phages were isolated and described, but only some of them were analyzed for their activity in AFB control. **Table 2** presents selected data regarding *P. larvae* phage application in bees. The available studies fed infected or healthy larvae with phages

TABLE 2 | Application of *P. larvae* phage or endolysin in bees.

Applied phages or endolysin	Source of phages	Mode of treatment	Results and recommendations	References
HB10c2 phage	Isolated from environment (the glue-like liquid of a beehive)	Infection during feeding, Bees fed with spores of *P. larvae* strain ERIC I DSM 7030 or ERIC II DSM 2530 at a concentration of 500 cfu/larva, phage was applied at a concentration of 50,000 pfu/larva	Phage did not cause bee mortality and did not disturb gut microbiota composition. However, phage therapy **was not efficient** in AFB treatment in infected larvae	Beims et al. (2015)
F, WA XIII phages	Phages isolated from *P. larvae* strain 2231	Infection during feeding. Larvae were infected with 1000 spores. Single phage (10^5–10^7 pfu/ml) or phages in cocktail (10^7 pfu/ml) were administered at day 0 or day 1.	Administered phages did not adversely affect survival of larvae. Phages applied before *P. larvae* NRRLB-3650 infection decreased larval mortality; the authors recommend **prophylactic use** of phage therapy against AFB	Ghorbani-Nezami et al. (2015)
PlyPa1A lysin	Isolated from *P. larvae* phage Xenia.	Larvae were infected with *P. larvae* B-3650 spores (1000 spores/larvae) with food were simultaneously treated with lysin at a concentration of 16 μg/ml.	The enzyme was active mainly against genotypes ERIC I. Do not disturb gut microbiota Larvae infected with spores and treated with single dose of the endolysin were rescued in 75%, which indicate the **therapeutic potential**.	Le Blanc et al. (2015)
Cocktail consisted of 7 phages: Xenia, Halcyone, Willow, Fern, Vadim, Harrison and Hayley	Phages isolated from: Xenia-infected hive, Halcyone-propolis, Willow-soil, Fern from wild strain 2231, Vadim- lipbalm, Harrison - soil Hayley- soil	Increasing amounts of food containing cocktail. Application within 7 days. Phage cocktail with a titer of 1.8×10^6 pfu/ml was applied before or after infection with spores.	Experiments indicated that **prophylactic administration** of a phage cocktail resulted in a higher survival of larvae than when applied as a treatment.	Yost et al. (2016)
Phage cocktail consisted of three phages (1, 5, 9)	Not known	Phage application with feeding. Phage cocktail applied to uninfected hives, hives in a mock-treated control group with a titer of 10^6 pfu/ml. After 2 weeks, 4 of the 5 hives in the control group were infected with AFB, while the five phage-treated hives remained healthy.	Phages did not cause deaths of healthy bees. The tested phages did not disturb the gut microbiota even after an overdose application and cocktail application, as observed in case of antibiotic application. **Protective and therapeutic effects** were observed in this study.	Brady et al. (2017)
PlyPl23 lysin	Isolated from genome of phage philBB_Pl23	Enzyme provided to larvae with feeding (diet containing 2.0 μM of enzyme).	The enzyme is safe and non-toxic for larvae which were observed during 5 days. It did not affect larvae development.	Oliveira et al. (2015)

or sprayed hive elements, e.g., combs. Ribeiro et al. (2019a) investigated in an *in vivo* study the ability of an active phage to penetrate larvae after *per os* administration of adult honey bees. T7 phage suspension in 50% (w/v) sucrose was applied and phage biodistribution was assessed in adult bees and larvae; phage penetration through food was confirmed in the larval midgut epithelium, which indicated that phages could be active at the site of *P. larvae* infection.

Unfortunately, phages, similarly as traditional antimicrobials (e.g., antibiotics), have the ability to destroy only vegetative forms of the causative agent of AFB, they are not able to destruct extremely infective spores.

Phage application in apiaries *in vivo* should be preceded by their detailed characterization (phage activity, lytic spectrum, life cycle parameters, genome sequences, phage stability under expected conditions at the site of application or infection) and testing their effectiveness *in vitro*. Ribeiro et al. (2019b) described the API480 phage isolated from a hive soil sample in Spain. The phage showed a broad lytic spectrum and was active against 69% of the tested field *P. larvae* strains *in vitro* representing both ERIC I and ERIC II genotypes. The integrase gene and lysogeny module were not identified in its genome. Examination of phage infection parameters revealed that adsorption was achieved several minutes after phage contact with bacterial cells, 85% of phage particles was adsorbed to its host after 35 min. The latent period lasted approximately 30 min., whereas its burst size was 3 pfu per bacterial cell. Furthermore, the phage was proved to be stable in high 50% (w/v) glucose concentration for 24 h and a slight reduction in phage titer (not statistically significant) was observed in homogenized larvae only after 24 h. These features together with the activity (despite the fact that the phage is temperate) observed *in vitro* suggested that it could be a good candidate for application in hives to treat or protect honey bees in field conditions.

Available studies indicated a possible protective effect for bees infected with these extremely resistant forms of bacteria.

The aforementioned effect of three *P. larvae* phages F, WA and XIII was studied *in vivo* by Ghorbani-Nezami et al. (2015) on larvae infected with NRRL B-3650 spores. The authors observed that the survival of larvae treated with phages (phage-treated control) as well as healthy larvae (negative control) was comparable and phages did not cause any deleterious effects. Based on these observations, the authors concluded that applying phages as prophylaxis (before infection with spores) provided better results than using them as therapeutic agents (applied after onset of infection symptoms). However, Brady et al. (2017) showed that phage cocktail active against *P. larvae* may be effective when used both as prophylaxis and therapeutic. Even an overdose cocktail did not exert adverse effects on the mortality of treated bees. The authors compared the efficacy of phage preparations and Tylan Soluble antibiotic, and a 19% decrease in hive health was observed in case of phage treatment applied as a therapeutic, compared to a 38% decrease caused by Tylan. Furthermore, phage application protected hives against *P. larvae* infection in 100%, whereas 80% of untreated hives were infected. These data indicated the potential of phages, especially in the prevention of AFB infection, and showed that a properly composed phage cocktail can be safer and more effective than antibiotics.

Endolysins and Their Potential Against *P. larvae*

Endolysins are enzymes encoded by bacteriophage genomes and used at the end of their life cycle to degrade peptidoglycan of the bacterial cell wall from within, resulting in cell lysis (Schmelcher et al., 2012). Phage lysins have many advantages, such as high specificity, stability, wide spectrum of activity or high efficiency, which allows their application as effective antimicrobials. Moreover, endolysins do not induce bacterial resistance, therefore, they are considered a promising alternative to phages (Loessner et al., 1995; Schmelcher et al., 2010). Oliveira et al. (2015) described the first *Paenibacillus larvae* endolysin PlyPl23 encoded by the genome of *P. larvae* siphovirus phiIBB_Pl23 with a high lytic potential. The enzyme had an *N*-acetylmuramoyl-L-alanine amidase catalytic domain. Compared to the source of the phage, the enzyme was proved to act specifically and lysed 100% of the tested vegetative forms of *P. larvae*, identified as belonging to different genotypes: ERIC I ERIC II and ERIC III (whereas phage phiIBB_Pl23 lysed only 80% of the tested strains). However, it was not active against *Bacilli* and *Lactobacilli* strains. Due to its high specificity, this enzyme can be applied specifically to eliminate *P. larvae* strains without interfering with bees' natural microbiota. An *in vitro* study that tested for 7 h heat-activated spores before and after germination showed that both dormant and germinating spores were not sensitive to lysin. It retained the activity in the pH range of 5–9 and after incubation with 25% and 50% sucrose; it was also stable in storage conditions (especially at −20°C for 22 weeks). In addition, lyophilization and reconstitution did not cause a loss of its activity. Interestingly, previous incubation with royal jelly increased the activity of PlyPl23, and a synergistic antibacterial effect between the enzyme and royal jelly was noted. The authors suggested that royal jelly could sensitize the cell wall of bacteria and enhance endolysin activity. *In vitro* lytic activity of the enzyme was also determined by measuring bacterial density in suspension. The authors observed that the activity of 0.2 μM endolysin reduced bacterial density (10^4 CFU mL^{-1}) to non-detectable level after only 30 min. *In vivo* safety tests confirmed that it was not toxic. Le Blanc et al. (2015) isolated lysin PlyPAlA with amidase activity from the genome of *P. larvae* phage Xenia (Le Blanc et al., 2015). Higher enzyme activity *in vitro* was observed against *P. larvae* strain with genotype ERIC I compared to ERIC III and IV. Exposure of *P. larvae* strains to 100 μg/ml of the enzyme reduced bacterial strains viability by 1–2 logs, whereas a dose of 700 μg/ml caused a 4-log decrease, indicating moderate bactericidal activity. Unfortunately, the enzyme did not kill the spores. A slight antibacterial effect was observed only in the case of germinating spores. After applying the enzyme to honey bee larvae, no disturbances were observed in the larval gut microbiota and one dose of lysin rescued up to 75% of larvae infected with spores. The above features indicate that phage lysin seems to be a better candidate than whole phages for preventing and eliminating *P. larvae* infection in bees. Comparison of their amino acid sequences showed high similarity, therefore the described enzymes are probably different variants of one phage protein. It is also possible that the already described *P. larvae* phages may encode previously unidentified lytic enzymes. It has been demonstrated that lysins may specifically bind spores, e.g., lysin LysPBC2 encoded by *Bacillus cereus* (Kong et al., 2019). These findings suggest the probability of isolating an endolysin with activity against spores produced by Gram-positive bacteria similar to *P. larvae*. The results have indicated that there is a need to further search for lysins with the above properties that may be encoded in *P. larvae* phage genomes. The modular structure of these enzymes creates the possibility of engineering proteins and constructing endolysins with new or improved properties. Enhanced lysin activity can be achieved by manipulating their functional domains, e.g., random or directed mutagenesis in the cell-binding domain (São-José, 2018; Kong et al., 2019), shuffling and fusion of catalytic domains with cell-wall binding domains of different origin and properties to obtain chimeric enzymes (chimeolysins), fusion of full-length lytic enzymes, domain deletion, addition or duplication, fusion to peptides, and combination of these methods (São-José, 2018). With respect to AFB sporicidal activity, it would be desirable for tailored enzymes that would penetrate the spore coat and then facilitate bond cleavage in peptidoglycan layers both in the core wall and spore cortex (Todar, 2009).

LIMITATIONS OF PHAGE USE FOR ELIMINATION OF AMERICAN FOULBROOD IN HONEY BEE

The use of phages or endolysins in the treatment of AFB in honey bee has not only advantages, but also some limitations. Phages are characterized by genomic plasticity. They are able not only to replicate, but also mutate in a specific bacterial

host. Additionally, they may induce expression of undesirable virulence factors, toxins and/or antibiotic resistance genes in the host (Łobocka et al., 2014). Most of the isolated phages against *P. larvae*, such as Davies, Jimmer1, Jimmer2, and PG1 are temperate because they encode integrases or transposases, which excludes their possible application in AFB treatment (Merrill et al., 2014; Stamereilers et al., 2018). Phage therapy against *P. larvae*, which is a spore-forming bacterium, may fail because of the possibility of spores protecting bacteria against lysis, which may lead to the development of reinfection (Beims et al., 2015).

Bacteria may already be resistant to phages. This situation means that bacterial susceptibility to selected bacteriophages should be tested every time whenever phages are planned to be utilized against these bacteria. An easier and faster solution is to prepare a cocktail that contains phages of different lytic spectra that can be active against wider host ranges, and application of this type of formulation may limit the probability of acquiring resistance to the applied phages (Merabishvili et al., 2018). Furthermore, bacterial collections on which the preparation activity will be tested should be regularly renewed with pathogenic strains from the area where phages are planned to be used to ensure that the phage preparation is active against currently or locally occurring bacterial strains (Merabishvili et al., 2018). Another possibility is to try to isolate new phages from materials from which pathogenic bacterial strains (that caused AFB) are isolated or various environmental samples that are collected.

There are many factors that may interfere with phage activity (when phages are intended as therapeutics), such as physico-chemical conditions, host physiological conditions, preparation composition or phage inability to penetrate and achieve high concentration at the site of infection (Jończyk-Matysiak et al., 2019). Phage efficiency may depend on its properties, structure and biology as well as therapeutic expectations, dose, manner of application, as well as modifications that can improve their activity and availability. The individual stability of the phage at different pH conditions is also an important issue, and this feature should be checked for each phage, as they tend to have different sensitivities to various physico-chemical conditions (Jończyk-Matysiak et al., 2019). These features should be taken into account and all phages contained in the preparation should be fully characterized. In addition to phages present in the preparation, it is also important to select proper additives that would protect phage activity and act as stabilizers. Moreover, the entire composition of the preparation intended for use in hives or to feed bees should be well tolerated by bees (taste and safety) and contain as few ingredients as possible.

Penetration of phages to the honey bee gut – the site of *P. larvae* infection – should be tested to ensure that the phages penetrate and are active at the site where AFB etiological agent is present. Therefore, spraying hive elements seems to be a less effective route of phage administration, resulting in a lower phage concentration on the hive surface. Despite the confirmed phage penetration at the site of infection, limitations of their action may be associated with low phage concentrations (as observed by Ribeiro et al., 2019a), not sufficient to reduce the count of *P. larvae* to prevent and cure AFB. Therefore, a high dose of phage particles should be provided and a method of phage protection against harmful hive-derived conditions is required (e.g., temperature, humidity, pH of larval food, persistence on the surface of hive elements). That is crucial to retain phage activity, as the conditions listed above vary in hive throughout the year. Bees have mechanisms to control the nest climate and specifically the brood area, but they depend on the prevailing weather conditions outside the nest and bee colony metabolism, which changes during the year (Stabentheiner et al., 2003, 2010; Tautz, 2008; Shaw et al., 2011; Cecchi et al., 2020). Moreover, phage preparations applied in the winter can remain deposited in wax patches even for several months and can cause significant reduction in phage activity. Under varied temperature conditions, phages may lose their activity during persistence in wax patches (Weinbauer, 2004); therefore, in order to protect phages applied in hives to control AFB, methods harmless to bees prolonging phage activity, such as encapsulation providing protection against harmful external factors, chemical or genetic modifications enabling extended phage activity, and addition of stabilizers protecting phages against their titer reduction should be considered.

It was demonstrated that phages could be inactivated during storage in larval food for a long time (Gochnauer, 1970). It was suggested especially in the case of larvae fed with phages and royal jelly that phage inactivation could be caused by the latter (low pH, composition), which could be one of the possible causes of failure of phage therapeutic effect in bees suffering from AFB (Yost et al., 2016; Ribeiro et al., 2019a). Hence, phages should be taken by larvae as fast as possible to ensure a neutral pH in gut conditions that guarantees phage stability. In addition to royal jelly, honey was proved to completely inactivate phages *in vitro* (Oliveira et al., 2017, 2018), which indicated that hive-derived products could influence phage activity in the hive environment.

Although *in vitro* studies on phages or their lysins are promising, phage activity depends on hive environment, larval gut conditions, phage individual features and its stability may significantly limit the effects of phage application *in vivo*. To protect phages and lysins against inactivation in hive and maintain phage stability during and after phage application, different methods may be used, e.g., encapsulation, addition of stabilizers, chemical or genetic modifications (mentioned in section "Endolysins and Their Potential Against *P. larvae*") to achieve prolonged activity or render phage or its protein extremely insensitive to environmental conditions.

Lysins appear to be effective against specific bacteria. However, studies using *P. larvae* phage lysins are scarce, which makes it difficult to infer from them meaningful conclusions. Since 2015, two lysins have been tested that (similarly to phages) were neither active against dormant nor germinating spores (Le Blanc et al., 2015; Oliveira et al., 2015). More studies should be conducted on a larger number of phage lytic enzymes in both laboratory and hive conditions, especially to allow comparison of the effects that form the full range bactericidal activity of

P. larvae-encoded lysins. Moreover, studies should be undertaken to develop methods allowing to make lysins more effective (especially against spores), active at the site of infection – in the larva's gut.

Currently, the trend of using natural products continues both in the treatment of bacterial infections and as part of a diet. Research by Naanwaab et al. (2014) showed that consumers declared to pay extra for bacteriophage-treated fresh product if it would improve their food safety. This indicated that consumers were not afraid of phage application in food, which can suggest that phage residues in bee products may also be acceptable.

Therefore, further research on both bacteriophages and lysins in the fight against AFB is required. Research in this area is still very limited and is urgently needed to save billions of honey bees all over the world.

CONCLUSION AND PERSPECTIVES

There is an urgent need for a safe, natural and effective product for the prevention and treatment of AFB in honey bees, whose application would not cause any adverse effects. There have been reports of isolation of bacteriophages active against *P. larvae*, and attempts of their use in AFB prevention and treatment. Published data indicated that the isolated specific phages showed the ability to lyse only the vegetative forms of *P. larvae* strains, and *in vivo* effects suggested that phages could be particularly useful in AFB prevention rather than treatment. Studies on phage-encoded endolysin are also promising. The use of phages or their enzymes in AFB therapy may reduce the need to eliminate hives by burning. Further research on phage application using different phage titers, different phage formulation compositions, forms of preparation (lysate or purified preparation), application (feeding, spraying), addition of different carriers both *in vitro* and *in vivo* during different stages of bee development is required. In addition, their application may reduce the risk of negative influence on the health of bees and consumers of bee products. Therefore, the possibility of using phages in treating AFB can bring great economic and environmental benefits as well as advantages for human health.

AUTHOR CONTRIBUTIONS

EJ-M conceptualized the study and drafted the main part of the manuscript. EP, BO, KH-S, KŚ-J, NŁ, DK, JN, PM, NB, FO, BW-D, AR, and AG contributed to the parts of the manuscript. EP and NŁ prepared figures. EJ-M, EP, and KH-S prepared tables. AG provided support and conceptual advice at all stages of manuscript preparation. All authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01913/full#supplementary-material

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OPINION
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The Unique Role That WHO Could Play in Implementing Phage Therapy to Combat the Global Antibiotic Resistance Crisis

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INTRODUCTION

Given the immensity of the global antimicrobial resistance (AMR) crisis, new avenues complementary to traditional antibiotics are urgently needed, particularly in developing countries where 90% of the predicted AMR deaths will occur (O'Neil, 2014). Bacteriophages (also known as phages) are a class of natural antimicrobials that were used, sometimes successfully (Abedon et al., 2011), before chemical antibiotics were discovered, and in recent years phages have been utilized to treat antibiotic-resistant infections (Kortright et al., 2019). Indeed, phage therapy is now undergoing a renaissance in industrialized countries, though their use has been little explored for low-income and lower-middle-income countries (LMICs). Rehabilitation of phage therapy represents both challenges and opportunities, which need to be dealt with at both local and global levels. Because of this, the World Health Organization (WHO) appears uniquely positioned to play a key role in the deployment of this atypical but promising technology as a means to combat antibiotic resistance.

THE PRESSING NEED IN DEVELOPING COUNTRIES

Phage therapy could be especially valuable for LMICs. In such settings, contaminated food or water, antimicrobial resistance, and also sanitary and hygiene problems are predisposing factors for bacterial infection. Moreover, malnutrition and HIV-mediated immunosuppression make their populations more vulnerable. There is thus an urgent need for supplementary treatment modalities in these areas, calling for the potential of phages to be unleashed in LMICs (Nagel et al., 2016).

As explained by Nagel et al. (2016), phages display several characteristics making them particularly suitable for LMICs. For instance, since phages are highly specific, they preserve commensal flora, which is a significant benefit for malnourished and immunocompromised individuals. In addition, phages can be easily isolated and produced locally using basic technological tools readily available in LMICs. The manufacturing of phages, excluding burden and costs incurred by regulatory and Good Manufacturing Practices (GMP) compliance, is inherently rapid and cost-effective. Also, the extemporaneous use of customized phage therapy medicinal products (PTMPs) could potentially minimize storage issues since cold-chain might not be needed. Phages could be complementary to vaccines since the quick killing activity of phages could buy enough time for the immune system to build a protective response to invading pathogenic bacteria. In addition to direct administration to people, phages can also be used to prevent food contamination

(Sulakvelidze, 2013; Moye et al., 2018). Finally, encouraging laboratories and biotech companies to develop new phage products, as part of the response to the immense public health needs of LMICs, could also contribute to the development of new market opportunities.

Accordingly, phage technology should be implemented in LMICs as part of a process aimed at transferring technology to local spheres and community-based management. And because of its public health experience and expertise in LMICs, WHO could serve as a reliable stakeholder, helping to ensure such knowledge transfer. In addition, WHO could play a central role in a global management plan to limit resistance development against phages by following pathogen evolution and diversity in LMICs. In this regard, the existing resources developed in the Global Antimicrobial Resistance Surveillance System (2015), including national and regional networks, capacity building tools, protocols, softwares, platforms etc…, might be exploited for collecting, sharing and analyzing data on phage resistance, therefore avoiding the unaffordable development and deployment of a phage specific surveillance system. On a broader front, phage therapy should be seriously considered among the global strategies to fight AMR, potentially as a part of the Global Action Plan on Antimicrobial Resistance (2015), which WHO helps lead.

THE PHAGE THERAPY ALTERNATIVE BUSINESS MODEL

Personalized medicinal products based on natural phages are not prone to strong patent protection and broad market distribution (Minssen, 2014; Todd, 2019). As such, phage therapy does not fit the market model that prevails for pharmaceuticals (Pirnay et al., 2011, 2012). Not surprisingly, the mainstream pharma industry has seemingly shown little interest in this area. Instead, this therapeutic practice is confined to not-for-profit phage therapy centers, hospitals or, to a lesser extent, some niche biotech companies. This limited commercial attractiveness poses a threat to the fundraising needed to meet the canonical pharmaceutical standards of quality, safety and efficacy. At the same time, however, this represents an opportunity for emerging countries, which might be motivated to include inexpensive phages in their medicinal practice. Under this scenario, WHO could be a valuable resource to compensate for the shortcomings of knowledge, technology, and regulatory skill associated with the limited incomes fueled by phage therapy. This makes the engagement of WHO, which operates as a not-for-profit international organization, positively essential for the mobilization of resources to be allocated to phage therapy deployment, especially in LMICs.

THE REGULATORY CHALLENGE FOR PHAGE THERAPY

Widely distributed phage therapy medications are made available through pharmacies in Russia and Georgia. These products are approved following regulatory processes that typically apply to ready-made preparations produced at a commercial scale (Chanishvili and Sharp, 2009). However, phage therapy may be regulated differently. Because of their narrow host range, phages could be used to specifically treat a given infectious event. Based on a "phagogram" establishing the susceptibility profile of the infecting bacteria, a customized preparation could be formulated by mixing suspensions of phages shown to lyse the infectious agent—and this could be done on an individual or local scale. However, the changing content of these preparations entails serious regulatory difficulties. Indeed, virtually all regulatory frameworks are meant to license industrially-prepared medicinal products that display a fixed qualitative and quantitative composition. In contrast, patient specific pharmaceutical preparations are usually considered as magistral formulas in the EU or compounded prescription drug product in the US. This led Belgium to implement a pragmatic approach for regulating phage therapy, based on the provision of the legislation on magistral preparation (Pirnay et al., 2018).

Another hindrance relates to the manufacturing requirements of PTMPs. Whereas, the need for a quality system is not questioned here, the GMP, as currently applicable for standard industry-made medicines, are typically out of reach for potential PTMP manufacturers. Indeed, full-blown GMP for PTMPs would imply major investments, out of proportion with the very small-scale production of named patient therapeutic phages. Instead, an *ad hoc* quality standard should be laid down, which ensures both the quality of the product and the safety for the patient, while allowing local productions at an affordable price. **Table 1** gives the minimum quality standards that should be applied in a pragmatic way to ensure that PTMPs are of suitable quality for their intended use.

In contrast to the prescriptive, strict, and sometimes dogmatic application of GMP standards by national authorities, the GMP audits conducted by WHO follow a more realistic approach. The patient is central in this exercise and there is no compromise on his or her safety and on the quality requirements of medicines. However, the WHO approach may take the form of a partnership aimed at strengthening the quality standards of the manufacturers rather than an authoritative inspection. Similar principles could be applied to the manufacture of PTMPs and instead of requiring strict adherence to standard GMP, *ad hoc* "Sound Manufacturing Practices" might be implemented for phages under the auspices of WHO.

WHO PREQUALIFICATION AS A TOOL FOR ASSURING QUALITY OF PHAGE STOCKS

In 1987, WHO launched the vaccines prequalification (PQ) programme. PQ was conceived as a service provided by WHO to United Nations (UN) procurement agencies such as UNICEF, which, following completion of the tender

TABLE 1 | Summary of the quality standards provided in the Belgian Phage Active Pharmaceutical Ingredient (API) monograph (Pirnay et al., 2018).

Process	Minimum quality standards
Quality system	Phage APIs are manufactured from phage seed lots under a quality system.
Production environment	The manufacturing of phage APIs takes place in an environment with specified air quality and cleanliness to minimize the risk of contamination. The effectiveness of these measures is validated and monitored. Where phage APIs are exposed to the environment during processing, without a subsequent microbial inactivation or removal process, an air quality with particle counts and microbial colony counts equivalent to those of Grade A, as defined in the current European Guide to Good Manufacturing Practice (GMP), Annex 1 and Directive 2003/94/EC is required with, if the system is not closed, a background environment at least equivalent to GMP Grade B in terms of particles and microbial counts.
Equipment and materials	Where equipment or materials affect critical processing or storage parameters (e.g., temperature, pressure, particle counts, and microbial contamination levels), they must be identified and subjected to appropriate monitoring, alerts, alarms, and corrective action, as required, to detect malfunctions and defects and to ensure that the critical parameters are maintained within acceptable limits at all times. All equipment with a critical measuring function is calibrated against a traceable standard if available. Maintenance, servicing, cleaning, disinfection, and sanitation of all critical equipment are performed regularly and recorded accordingly. Standard Operating Procedures (SOPs) detail the specifications for all critical materials and reagents. In particular, specifications for culture media, additives (e.g., solutions) and packaging materials are defined. Where applicable, reagents and materials meet compendial requirements and/or documented specifications and the requirements of Regulation 2017/745 of the European Parliament and of the Council of 5 April 2017 on medical devices and Regulation 2017/746 of the European Parliament and of the Council of 5 April 2017 on *in vitro* diagnostic medical devices. Animal component free culture media and additives should preferably be used. Host bacteria used in the manufacturing process are as safe (or least pathogenic) as possible. Non-lysogenic bacterial strains are used, if possible.
Purification	Purification methods (e.g., filtration and affinity chromatography) need to be applied to minimize the content of harmful bacterial or culture medium components (e.g., bacterial endotoxins and animal products).
Preservation/Storage	Phage seed lots and phage APIs need to be stored using validated preservation/storage methods (e.g., cooling, cryopreservation, and freeze-drying).
Release testing (by a Belgian Approved Laboratory)	**Phage seed lots.** • **Phage identification.** State of the art DNA or RNA sequencing and genome analysis. When reliable *in silico* morphology prediction is not possible, phage morphology should be determined by electron microscopy. • **Phage enumeration.** The phage enumeration of the phage seed lot should be determined using an appropriate method (e.g., pfu determination or qPCR). • **Phage purity.** Absence of adventitious agents (e.g., other phages, bacteria, or viruses) should be demonstrated using an appropriate method, unless otherwise justified (e.g., virus testing may be omitted if no human or animal origin is used). • **Detection of genetic determinants conferring toxicity, virulence, lysogeny, and antibiotic resistance.** State of the art DNA or RNA sequencing and genome analysis. **Phage APIs.** All tests are performed under appropriate quality standards (e.g., ISO17025). • **Phage identification.** The phage strain of a phage API is determined using a validated or qualified phage identification test (e.g., specific PCR, qPCR). • **Quantitative assessment of phages.** The potency of the phage API is determined using a validated or qualified assay (e.g., phage-specific qPCR). • **Quantitative bioburden determination (EP 2.6.12).** The total aerobic microbial count is determined using the official Ph.Eur. method, or where justified and authorized, using a validated alternative method. Phage APIs are required to contain $\leq$10 cfu/100 ml or g. • **Bacterial endotoxins (EP 2.6.14).** The test for bacterial endotoxins is used to detect and quantify endotoxins of gram-negative bacterial origin using amoebocyte lysate from horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). The endotoxin limit depends on the final therapeutic product (magistral preparation) and its route of administration and is stated in the individual monograph according to compendial requirements. The maximal dose administered by the intended route per hour should not contain sufficient endotoxin to cause a toxic reaction. For instance, as stated in EP 5.1.10, the maximum dose for intravenous injection is 5 Endotoxin Units (EU)/kg/h. • **Potentiometric determination of pH (EP 2.2.3).** The pH should conform to the pH specifications set forth in the individual monograph, usually 6.0–8.0 pH. • **Water content (EP 2.5.12 or 2.5.32).** Dried phage APIs are tested for water content. The maximum water content is 3.0 per cent m/m, unless otherwise stated in specific monograph (e.g., APIs intended for oral lyophilisates). • **Impurities.** Process-related impurities should be quantified and qualified. Appropriate acceptance criteria should be set up such that the amounts of impurity intake are consistently below levels that are demonstrated to be safe.
Shelf life	Phage quantity (using a stability indicative method), bioburden, pH and, where relevant, water content are periodically determined. The shelf life is the time period during which phage quantity, bioburden, pH, and where relevant water content, of the API remain within the specified limit thresholds.
Labeling	The label states all information necessary to identify the content and specific instructions or warnings for administration, storage and disposal (e.g., phage identity and quantity, host bacteria, storage conditions, the production date, the expiration date, instructions for reporting serious adverse reactions and/or events, and instructions how to dispose of unused (expired) products).
Surveillance	The clinical use of phage API based magistral preparations must be surveyed and reported, including possible adverse events and reactions associated with their use. A centralized reporting system and a register for therapeutic phage applications are warranted.

process, will eventually select or "qualify" the bidder(s) (Dellepiane and Wood, 2013). Initially, PQ was limited to the testing of vaccine lots, review of summary lot protocols, and inspection of manufacturing facilities. Today, PQ has become a hallmark of quality and trust, which has been expanded in various ways. First, the scope of vaccines PQ has been reinforced since it now includes a scientific review of quality, safety, and efficacy evidences, and the participation of the national regulatory authority (NRA) of the vaccine manufacturing country. Second, the PQ process has been extended to other products and services such as medicines, active pharmaceutical ingredients (APIs), *in vitro* diagnostics, and vector control products. Third, the PQ process broadened its customer base. Indeed, whereas PQ was initially intended to UN procurement agencies, today, national governments, international organizations, public-private partnerships and NGOs can also make use of this service. Finally, it is worth mentioning that PQ goes beyond a strict evaluation exercise since it provides technical assistance to product manufacturers, training, capacity building and benchmarking of NRA, advisory activities to stakeholders, as well as opportunities for collaboration between regulators.

In contrast to NRAs, WHO is not a regulatory authority. As such, it is not restrained by burdensome lawmaking processes and, consequently, it can enjoy some flexibility in the implementation of procedures, especially to address critical global health issues. The PQ of vector control products nicely illustrates WHO's capability to implement a procedural response for controlling products that are otherwise not regulated. It is precisely the situation that PTMPs are facing today. Phage therapy, as a promising response against antimicrobial resistance, offers an evident public health potential. However, because of the legal conundrum it elicits, only a handful of countries set up a regulatory framework for PTMPs. Unquestionably, a WHO PQ of phage stocks would provide a major boost to the controlled and safe use of phage therapy. Also, phage PQ would particularly benefit countries with poor regulatory oversight, which are also those that most urgently need PTMPs.

Since the regulatory requirements of phage therapy are expected to primarily focus on the phage stocks (and not on the finished product), WHO PQ of APIs appears as a particularly relevant model. Typically, PQ programmes aim at assessing the suitability of chemical APIs for use in the manufacture of finished pharmaceutical products (FPP) (WHO, Essential Medicines and Health Products: Prequalification of Medicines[1]. It involves evaluation of data relating to their quality, as well as inspection of the relevant manufacturing site(s). This service is intended for FPP manufacturers, which can then rely on potential sources of good quality APIs manufactured in compliance with GMP. This scheme could easily be transposed to the PQ of phage stocks, making sure that the responsible pharmacist is confident about the quality of the stocks to be used as active ingredients for the formulation of PTMP.

[1]https://extranet.who.int/prequal/content/active-pharmaceutical-ingredients-0

VERTICAL COOPERATION AS A MUST FOR SUCCESSFUL PHAGE THERAPY DEPLOYMENT

Phage therapy can only realize its full potential through the implementation of vertical synergies involving local, national and global stakeholders.

Phage therapy is intrinsically best served by local applications, for at least three reasons. First, there is a greater chance of finding an active phage in the local environment of the bacteria that it is meant to target. Second, phage therapy should best be prescribed bedside (for individuals) or at local scale (for regional populations), following the interpretation of a phage susceptibility test of the infecting bacteria (phagogram), ideally following specialist microbiologist advice (Henein, 2013). Third, bacterial infections usually require a quick response: the closer to the phage stock, the faster the response. As such, it makes much sense to produce PTMPs close to their point of use. This perfectly fits with the initiative of six United Nations agencies and international organizations, including WHO, which co-signed the Interagency Statement on Promoting Local Production of Medicines Other Health Technologies (2019). During the launch event held in Geneva on 24 May 2019, the Director-General of WHO, Dr. Tedros Adhanom Ghebreyesus, said that "Local production has obvious benefits for health by creating a reliable and affordable supply of essential medicines and other health products… It also creates jobs and contributes to economic growth… but realizing the promise of local production is not straightforward" (World Health Organization, 2019). Indeed, as reported in the accompanying press statement, "local production of quality-assured health products requires a holistic approach that considers policy coherence, regulatory systems strengthening, access to finance for sustainable production, a careful assessment of the business case, development of skilled human resources, access to technology for production and needs-based innovation, creation of investment incentives, and other factors, to enable manufacturers to comply with international quality standards, be competitive and engage in sustainable manufacturing" (UNAIDS, 2019). Whereas, part of this endeavor lies in the hands of national authorities, all these achievements cannot be fulfilled by local governments alone, especially since in many LMICs the capacity of both local manufacturers to produce and supply quality medical products and the NRAs to ensure quality, efficacy, and safety are insufficient. This is where the involvement of international organizations could make a difference. Considering that the manufacturing technology of phages is neither cutting-edge nor expensive, that these therapeutics follow a non-traditional business model and that, ideally, they should be produced *in situ*, PTMPs emerge as a model for sustainable and qualitative local productions of medicines, as envisioned and promoted by WHO.

Phage therapy also requires global action. Whereas, the physical management of the phage seed stocks and collections should remain in the hands of local practitioners, the phage information (targeted bacterial species/strains/isolates, genotypic, and phenotypic characterization, production host

strains, etc.) should be gathered in a publicly available central database for at least two reasons. First, ensuring open access to such information would enable quick response to patients' needs—and timely response can save the lives of those with bacterial infections. Second, establishing an exhaustive sequence database would make it possible to implement a ground-breaking and extremely promising approach to phage therapy. In this cutting-edge process, phage genome sequences, and their phenotypic characteristics (i.e., target bacteria) could be analyzed by deep learning algorithms to instantly establish a match between the infecting bacteria identified bedside and the curative phage. In the future, based on the matching phage sequence available in the database, the corresponding phage genome could even be synthesized locally in order to produce a synthetic phage on site, which could be swiftly administered to the patient. Collecting the data of phage and matching bacterial genome sequences from a large number of laboratories would be greatly enhanced if it takes place under the auspices of a major and financially disinterested public health player, which inspires confidence and assures long term stability (Pirnay, 2020), in accordance with the terms of the Nagoya protocol. WHO rises up as a reliable supportive guarantor of such an undertaking.

Additionally, surveillance and pharmacovigilance information should be reported centrally and integrated in this database. Where applicable, phage stocks' "labeling" information should be updated with the safety and efficacy information collected through pharmacovigilance monitoring.

More generally, a global health strategy should be implemented to avoid the past mistakes, such as those related to the unconsidered use of antibiotics. Since the administration of phages as antimicrobial agents might result in acquired resistance, integrated use policy, responsible management, as well as good practices and guidelines should be implemented globally.

Phage therapy implementation thus requires global, national and local initiatives. As such, WHO is particularly well-placed to drive this process. Being an international organization, WHO is in a position to implement health practices at a global level. Thanks to its network of regional and country offices and several decades of interaction with NRAs, WHO may also exert its influence at both national and local levels.

TO CONCLUDE

Implementation of phage therapy is accompanied by considerable difficulties, but in the face of the 10 million people who will likely die annually by 2050 due to AMR, public health policy makers cannot afford to exclude promising tools in the struggle against infections caused by multidrug resistant bacteria (O'Neil, 2014; De Vos and Pirnay, 2015). WHO is challenged to play a key role in this process, which would primarily benefit LMICs. WHO possesses the medical, scientific and programmatic capabilities to meet this challenge, and, with the PQ program, it already harbors a powerful procedural tool, which might suitably regulate this unorthodox therapeutic practice.

It is our hope that this call will be heard.

AUTHOR CONTRIBUTIONS

AF conceived of the overall topic and defined the contents of the manuscript. AF, TN, CF, and J-PP wrote the text. GV, DD, and MM reviewed the manuscript. All authors read and approved the final manuscript.

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Evaluation of Staphylococcal Bacteriophage Sb-1 as an Adjunctive Agent to Antibiotics Against Rifampin-Resistant *Staphylococcus aureus* Biofilms

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Rifampin plays a crucial role in the treatment of staphylococcal implant-associated infection, as it is the only antibiotic capable of eradicating *Staphylococcus aureus* biofilms. However, the emergence of rifampin resistance strongly limits its use. Combinatorial therapy of antibiotics and bacteriophages may represent a strategy to overcome the resistance. Here, we evaluated the activity of staphylococcal bacteriophage Sb-1 in combination with different antibiotics against the biofilms of 10 rifampin-resistant *S. aureus* clinical strains, including MRSA and MSSA. *S. aureus* biofilms formed on porous glass beads were exposed to antibiotics alone or combined with Sb-1 simultaneously or staggered (first Sb-1 for 24 h followed by antibiotic). Recovered bacteria were detected by measuring growth-related heat production at 37°C (isothermal microcalorimetry) and the biofilm eradication was assessed by sonication of beads and plating of the resulting sonication fluid. Minimum biofilm eradication concentration (MBEC) was defined as the lowest concentration of antibiotic required to kill all adherent bacteria, resulting in absence of growth after plating the sonication fluid. Tested antibiotics presented high MBEC values when administered alone (64 to > 1,024 μg/ml). The simultaneous or staggered combination of Sb-1 with daptomycin showed the highest activity against all MRSA biofilms, whereas the exposure to Sb-1 with vancomycin showed no improved anti-biofilm activity. Staggered administration of Sb-1 and flucloxacillin, cefazolin, or fosfomycin improved the antibiofilm activity in four out of six MSSA, whereas simultaneous exposure exhibited similar or lesser synergy. In conclusion, the combinatorial effect of Sb-1 and antibiotics enabled to eradicate rifampin-resistant *S. aureus* biofilms *in vitro*.

Keywords: rifampin-resistant *Staphylococcus aureus*, bacterial biofilm, antibiotic–bacteriophage combination, phage therapy, synergism, isothermal microcalorimetry

INTRODUCTION

Staphylococcus aureus is one of the most common organisms causing implant-associated infections, such as periprosthetic joint infections (PJI), fracture-related infections (FRI), or spinal implant-associated infections (Tong et al., 2015). The pathogenesis involves the colonization of the device by microorganisms leading to the formation of biofilm on the surface of the implant, which makes the treatment of these infections challenging. Optimal treatment implies debridement and retention of the implant (in acute infections) or debridement with removal of devitalized material and exchange of implant that contain mature biofilm (in chronic infections) (Izakovicova et al., 2019). In both clinical situations, eradication of the biofilm with prolonged administration of biofilm-active antibiotics is required (Sendi et al., 2008).

The treatment of implant-associated infections due to *S. aureus* consists of initial intravenous antibiotic therapy, including nafcillin, oxacillin, flucloxacillin, cefazolin, or fosfomycin against methicillin-susceptible *S. aureus* (MSSA) and vancomycin, daptomycin, or fosfomycin against methicillin-resistant *S. aureus* (MRSA). In addition, rifampin is added to treat staphylococcal infections in patients who undergo debridement with retention or implant re-implantation in one- or two-stage exchange (Berbari et al., 2020). Rifampin should be co-administered with another active antibacterial agent since, otherwise, rifampin resistance emerges rapidly (Hoiby et al., 2015). With increased rifampin use, rifampin-resistant staphylococcal strains are increasing worldwide, representing an important concern. For example, in China, the rifampin resistance in MRSA isolates increased from 15.5 to 50.2% within 4 years (2004–2008) (Wang C. et al., 2019). Against rifampin-resistant mutants, rifampin has no biofilm activity *in vitro* or *in vivo* (Croes et al., 2010). Thus, alternative antimicrobial agents were investigated (e.g., daptomycin, fosfomycin, and dalbavancin), but none has shown biofilm activity *in vivo*. Another alternative is a combination of antibiotics with lytic bacteriophages. Lytic bacteriophages might exhibit rapid bactericidal activity, biofilm degradative properties, and the ability to enhance antibiotic activity (Tkhilaishvili et al., 2018) and are therefore considered as alternative strategies combating bacterial infections (Reardon, 2014).

Phage Sb-1 is one of the best characterized and fully sequenced lytic *staphylococcal* phage developed as an anti-infective therapy for human application by the Eliava Institute in Georgia (Kutateladze and Adamia, 2008). Its genome does not contain any bacterial virulence-associated genes, making it suitable for antimicrobial therapy (Kvachadze et al., 2011). Moreover, Sb-1 has been successfully used during the former Soviet Union to treat *S. aureus* infections in different patients (Sulakvelidze et al., 2001). However, there are limited numbers of *in vitro* and *in vivo* studies published regarding the activity of phage–antibiotic combination against *S. aureus* strains. Our previous study showed a good synergistic activity of phage Sb-1 and antibiotics against MRSA ATCC 43300 (Tkhilaishvili et al., 2018). In this study, we evaluated the efficacy of different classes of antibiotics (vancomycin, daptomycin, fosfomycin, gentamicin, flucloxacillin, cefazolin, and rifampin) alone or in combination with Sb-1, by either simultaneous or staggered application, against 10 rifampin-resistant *S. aureus* (RRSA) clinical strains (four MRSA and six MSSA) and the MRSA ATCC 43300 and MSSA ATCC 29213 laboratory strains.

MATERIALS AND METHODS

Bacteria and Bacteriophage

Ten RRSA clinical isolates collected between 2015 and 2019 were included in this study. The clinical isolates were used from the biobank collection, which is part of the prospective institutional PJI cohort. The study was approved by the institutional ethical committee (EA1/040/14) and was conducted in accordance with the most recent iteration of the Declaration of Helsinki. According to the ethical approval, participants' informed consent was waived, and all data were pseudonymized. Moreover, MRSA ATCC 43300 and MSSA ATCC 29213 laboratory standard strains were used in this study. Bacteria were stored at −80°C using a cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Canada). The staphylococcal phage Sb-1 was supplied by the Eliava Institute (Tbilisi, Georgia) and stored at 4°C.

Antimicrobial Agents and Susceptibility Testing

Vancomycin (0.5 g, Hexal, Holzkirchen, Germany), daptomycin (0.5 mg, Novartis Pharma Schweiz, Basel, Switzerland), fosfomycin (5 g, InfectoPharm, Heppenheim, Germany), gentamicin injectable solution (40 mg/ml, Ratiopharm, Ulm, Germany), flucloxacillin (2 g, Stragen Pharma, Bad Homburg, France), cefazolin (2 g, MIP Pharma, Blieskastel-Niederwuerzbach, Germany), and rifampin (6 g, Sandoz Pharmaceuticals, Steinhausen, Switzerland) were provided from the respective manufacturers.

MIC for each antibiotic was determined by the broth macrodilution assay (BMD) in brain heart infusion broth (BHI; BD, Le Pont de Claix, France). An inoculum of approximately 5×10^5 CFU/ml was used. Two fold serial dilutions of each antibiotic were prepared in sterile polystyrene round-bottom tubes to a final volume of 1 ml in inoculated medium and incubated for 24 h at 37°C. The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible growth. BHI broth medium was supplemented with calcium chloride (40 μg/ml) and glucose 6-phosphate (25 μg/ml) when testing daptomycin and fosfomycin, respectively. All experiments were performed in triplicates.

The bacterial susceptibility to Sb-1 was evaluated in terms of efficacy of plating (EOP) as previously described (Wang et al., 2016). The EOP value was calculated as the ratio between the plaque-forming units (PFU) on the tested clinical strains with respect to the MRSA ATCC 43300 strain, defined as the host

bacterium (EOP = phage titer on test bacterium/phage titer on host bacterium). EOP values of 0.5–1 were ranked as "high" efficiency; 0.2–0.5 as "medium" efficiency; 0.001–0.2 as "low" efficiency; 0.0 was considered as not effective against the target strain (Viazis et al., 2011).

Evaluation of Antibiofilm Activity by Isothermal Microcalorimetry and Sonication/Colony Counting

The antibiofilm activity of single antibiotics and phage–antibiotic combination was determined by isothermal microcalorimetry (IMC), as previously reported (Tkhilaishvili et al., 2018). Briefly, biofilm formation was conducted by incubating porous glass beads (ROBU, Hattert, Germany) in inoculated BHI media at 37°C for 24 h. Beads where then washed (3x) with sterile 0.9% NaCl to remove planktonic cells and exposed to fresh BHI containing antibiotic. After 24 h of incubation at 37°C, beads were rinsed (3x) with 0.9% saline and inserted in microcalorimetry ampoules containing 3 ml of fresh BHI and introduced into the calorimeter. The minimum biofilm bactericidal concentration (MBBC) was defined as the lowest concentration of antibiotic that led to the absence of heat production after 48 h of incubation at 37°C. The effect of combined treatment (antibiotic + Sb-1) was evaluated by either simultaneous or staggered application, of 10^6 PFU/ml Sb-1 phage and sub-MBBC concentrations of antibiotics. By simultaneous application, biofilms were exposed to antibiotics and Sb-1 during 24 h at 37°C. By staggered application, biofilms were exposed first to Sb-1 for 24 h and then to antibiotic for a further 24 h at 37°C. Evaluation of a staggered application of antibiotic followed by phage was discarded based on the unfavorable results observed in previous studies (Kumaran et al., 2018; Tkhilaishvili et al., 2018).

For samples where no heat production was detected, the complete biofilm eradication was determined by CFU counting of the sonicated beads after the microcalorimetric assay, and the minimum biofilm-eradicating concentration (MBEC) was defined as the lowest concentration of antibiotic required to kill all adherent bacteria, resulting in absence of any growth after plating of the sonication fluid (detection limit: <20 CFU/ml) (Gonzalez Moreno et al., 2019). All experiments were performed in triplicates.

The effect of phage–antibiotic combinations against biofilms was assessed as in a previous study (Ryan et al., 2012), determining the $MBEC_{phage}/MBEC_{alone}$ ratio, where $MBEC_{phage}$ corresponds to the obtained MBEC value of an antibiotic tested in combination with the phage, and the $MBEC_{alone}$ represents the obtained MBEC value of the same antibiotic when tested alone. Synergy was defined as a ratio $\leq$ 0.25, which correlated with a reduction of more than $2xMBEC_{alone}$. We combined and tested only concentrations of antibiotic that could reveal a synergistic effect with Sb-1 based on the MBEC values of the single antibiotic to be combined (antibiotics presenting an $MBEC_{alone}$ > 1,024 μg/ml were tested in combination with Sb-1 at increasing concentrations up to 256 μg/ml).

RESULTS

Bacterial Susceptibility to Conventional Antibiotics and Sb-1

The antimicrobial activity of antibiotics against planktonic and biofilm *S. aureus* was determined by BMD and by plating of sonication fluid, respectively. The obtained MIC and MBEC values are summarized in **Table 1**. Additionally, the MBBC values assessed by IMC and the effect of Sb-1 against the biofilm of both ATCC strains are shown in **Supplementary Figures S1, S2**.

Both ATCC strains were susceptible to all antibiotics according to the EUCAST breakpoints (EUCAST, 2020), except for MRSA ATCC 43300 that was resistant to gentamicin. The 10 RRSA strains were susceptible to all antibiotics, besides for MRSA4, resistant to fosfomycin, and MSSA5, resistant to gentamicin. All tested strains were susceptible to higher concentrations of antibiotics (ranging from 64 to > 1,024 μg/ml) when grown as biofilms if compared to the MIC values obtained for planktonic bacteria.

The antibiofilm activity of different antibiotics against the ATCC strains was evaluated by monitoring for 48 h the heat produced by biofilm bacteria still viable on the beads (after the exposure to the antibiotics) re-inoculated in fresh medium (**Supplementary Figure S1**). On the one hand, MRSA ATCC 43300 was susceptible to daptomycin and rifampin at concentrations of 128 and 256 μg/ml, respectively, whereas MSSA ATCC 29213 showed susceptibility to gentamicin and rifampin at concentrations of 512 and 256 μg/ml, correspondingly. The rest of the antibiotics, tested up to 1,024 μg/ml, showed no inhibition of heat flow production on the corresponding strain, indicating no antibiofilm activity despite the presence of high concentrations of antibiotic.

The exposure of the biofilm from both ATCC strains during 24 h to Sb-1 revealed a distinct effect on each strain. A remarkable reduction but not complete inhibition of the heat-flow production compared to the heat-flow produced by the growth control could be observed with the treated MRSA strain, whereas almost no difference between control and treated sample was observed for MSSA (**Supplementary Figure S2**). All *S. aureus* strains were susceptible to Sb-1 infection, showing EOP ratios ranging from 0.3 to 0.9 (**Supplementary Table S1**), indicative of a high lytic activity (EOP 0.5–1) of Sb-1 against most strains.

Evaluation of Phage–Antibiotic Combinations Against ATCC Strains

The synergistic effect of simultaneous (**Figure 1**) and staggered (**Figure 2**) phage–antibiotic combinations against biofilm of both ATCC strains was investigated by IMC. Additionally, the presence of viable bacteria attached to the beads after calorimetry of those samples showing no heat production was evaluated by colony counting after bead sonication and plating of the sonication fluids. The obtained MBEC values as well as the calculated $MBEC_{phage}/MBEC_{alone}$ ratios are summarized in **Table 2**.

Among all simultaneously tested phage–antibiotic combinations against MRSA ATCC 43300, only the exposure of

TABLE 1 | Antimicrobial susceptibility of planktonic (MIC) and biofilm (MBEC) *Staphylococcus aureus* strains determined by conventional broth macrodilution assay and sonication/colony-counting.

Antibiotic	**VAN**		**DAP**		**FOF**		**GEN**		**RIF**	
MRSA strains	**MIC**	**MBEC**	**MIC**	**MBEC**	**MIC**	**MBEC**	**MIC**	**MBEC**	**MIC**	**MBEC**
MRSA ATCC 43300	1	>1,024	0.5	128	8	>1,024	64(R)	>1,024	0.008	256
MRSA1	1	>1,024	0.5	64	4	>1,024	0.5	256	32(R)	>1,024
MRSA2	2	>1,024	1	128	4	>1,024	0.5	512	1(R)	>1,024
MRSA3	1	>1,024	0.5	64	128(R)	>1,024	0.5	512	32(R)	>1,024
MRSA4	1	>1,024	1	128	4	>1,024	0.5	>1,024	4(R)	>1,024
Antibiotic	**FLU**		**CFZ**		**FOF**		**GEN**		**RIF**	
MSSA strains	**MIC**	**MBEC**	**MIC**	**MBEC**	**MIC**	**MBEC**	**MIC**	**MBEC**	**MIC**	**MBEC**
MSSA ATCC 29213	0.5	>1,024	0.5	>1,024	4	>1,024	1	512	0.016	256
MSSA1	0.25	1,024	0.25	>1,024	16	>1,024	0.5	512	1(R)	>1,024
MSSA2	0.5	1,024	0.5	>1,024	4	>1,024	0.5	512	32(R)	>1,024
MSSA3	0.5	1,024	0.5	>1,024	8	>1,024	0.5	>1,024	32(R)	>1,024
MSSA4	0.25	>1,024	0.25	>1,024	8	>1,024	0.5	>1,024	1(R)	>1,024
MSSA5	0.5	512	0.5	>1,024	16	>1,024	8(R)	>1,024	2(R)	>1,024
MSSA6	0.5	>1,024	0.25	>1,024	4	>1,024	0.5	1,024	1(R)	>1,024

MIC and MBEC concentration values are expressed in μg/ml. VAN, vancomycin; DAP, daptomycin; FOF, fosfomycin; GEN, gentamicin; RIF, rifampin; FLU, flucloxacillin; CFZ, cefazolin; R, resistance against the antibiotic according to EUCAST.

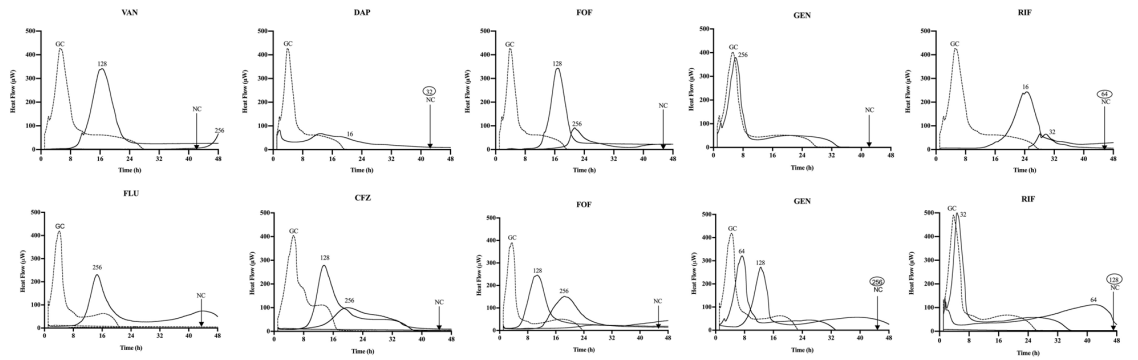

FIGURE 1 | Microcalorimetry analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 (upper row) and methicillin-susceptible *S. aureus* (MSSA) ATCC 29213 (bottom row) biofilms treated simultaneously with Sb-1 phage and sub-minimum biofilm bactericidal concentration (MBBC) concentrations of antibiotics. Each curve shows the heat produced by viable bacteria present in the biofilm after 24 h of phage–antibiotic treatment. Numbers represent concentrations (in μg/ml) of vancomycin (VAN), daptomycin (DAP), fosfomycin (FOF), gentamicin (GEN), rifampin (RIF), flucloxacillin (FLU), and cefazolin (CFZ). Circled values represent the MBBC, defined as the lowest antimicrobial concentration leading to absence of bacterial regrowth after 48 h. GC, growth control; NC, negative control. Data of a representative experiment are reported.

biofilm to Sb-1 and sub-MBBC concentrations of daptomycin or rifampin showed a synergistic effect. In contrast, the strongest synergistic effect was observed by staggered exposure of MRSA ATCC 43300 to Sb-1 and sub-MBBC concentrations of vancomycin or daptomycin, showing the lowest $MBEC_{phage}/MBEC_{alone}$ ratios, followed by fosfomycin and rifampin (**Table 2**), whereas no synergistic effect was observed with gentamicin, possibly due to the resistance profile of this strain toward gentamicin.

No synergistic effect was observed by the phage–antibiotic combinations against MSSA ATCC 29213, either simultaneous or staggered. Overall, when MRSA ATCC 43300 biofilm was first exposed to Sb-1 during 24 h prior to the exposure to sub-MBBC concentrations of antibiotics, a higher delay and/or reduction of heat-flow production was obtained compared to the heat-flow produced when biofilm was exposed to simultaneous phage/antibiotic combinations at the same antibiotic concentrations, whereas this effect was not as noteworthy in the case of MSSA ATCC 29213.

Evaluation of Phage–Antibiotic Combinations Against Clinical Rifampin-Resistant *Staphylococcus aureus* Strains

The ability of phage–antibiotic combinations to eradicate the biofilm of 10 clinical rifampin-resistant MRSA and MSSA

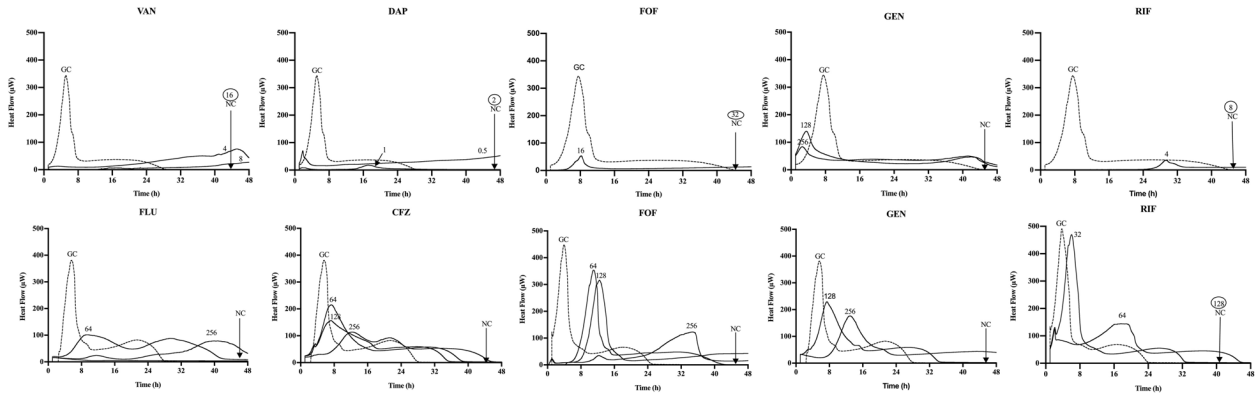

FIGURE 2 | Microcalorimetry analysis of MRSA ATCC 43300 (upper row) and MSSA ATCC 29213 (bottom row) biofilms after staggered application of Sb-1 phage for 24 h followed by 24 h exposure to sub-MBBC concentrations of antibiotics. Each curve shows the heat produced by viable bacteria present in the biofilm after phage–antibiotic treatment. Numbers represent concentrations (in μg/ml) of vancomycin (VAN), daptomycin (DAP), fosfomycin (FOF), gentamicin (GEN), rifampin (RIF), flucloxacillin (FLU), and cefazolin (CFZ). Circled values represent the MBBC, defined as the lowest antimicrobial concentration leading to absence of bacterial regrowth after 48 h. GC, growth control; NC, negative control. Data of a representative experiment are reported.

TABLE 2 | Antibiofilm effects of simultaneous or staggered phage–antibiotic combinations.

Antibiotic	Simultaneous exposure		Staggered exposure	
	MBEC (μg/ml)	Ratio (interpretation)	MBEC (μg/ml)	Ratio (interpretation)
MRSA ATCC 43300				
VAN	>256	> 0.25 (NS)[a]	16	0.015 (S)
DAP	32	0.25 (S)	2	0.015 (S)
FOF	>256	> 0.25 (NS)[a]	32	0.031 (S)
GEN	>256	> 0.25 (NS)[a]	>256	> 0.25 (NS)[a]
RIF	64	0.25 (S)	8	0.031 (S)
MSSA ATCC 29213				
FLU	>256	> 0.25 (NS)[a]	>256	> 0.25 (NS)[a]
CFZ	>256	> 0.25 (NS)[a]	>256	> 0.25 (NS)[a]
FOF	>256	> 0.25 (NS)[a]	>256	> 0.25 (NS)[a]
GEN	256	0.5 (NS)	>256	> 0.25 (NS)[a]
RIF	128	0.5 (NS)	128	0.5 (NS)

VAN, vancomycin; DAP, daptomycin; FOF, fosfomycin; GEN, gentamicin; RIF, rifampin; FLU, flucloxacillin; CFZ, cefazolin; S, synergism; NS, no synergism. [a]MBEC value above 1/4xMBEC$_{alone}$ (considering MBEC$_{alone}$ equal to 1,024 μg/ml), thus MBEC$_{phage}$/MBEC$_{alone}$ ratio is interpreted as >0.25 (NS).

strains was directly evaluated by sonication/colony-counting as described previously, and results are shown in **Table 3**.

Among four MRSA isolates, a synergistic effect was observed for all strains after exposure to Sb-1/daptomycin combination (either simultaneously or staggered), in three strains (75%) when exposure to Sb-1/gentamicin staggered combination and in two (50%) strains exposed to staggered Sb1/fosfomycin or to simultaneous Sb-1/gentamicin combination. No synergistic effect was observed when the biofilm of tested clinical strains was exposed to either simultaneous or staggered Sb-1/vancomycin combination, in contrast to the finding with MRSA ATCC 43300 strain.

Among six MSSA isolates, synergistic effect was observed in four strains (67%) after staggered exposure to Sb-1/flucloxacillin or to Sb-1/cefazolin, and in three strains (50%) with simultaneous Sb-1/flucloxacillin combination. Only staggered but not simultaneous Sb-1/fosfomycin combination revealed a synergistic effect against four strains (67%), whereas simultaneous or staggered Sb-1/gentamicin combination showed synergism against three strains (50%). None of the tested simultaneous or staggered phage/antibiotic combinations presented an improvement in the antimicrobial activity compared to the action of each antimicrobial agent alone against MSSA5 biofilm.

Moreover, no synergism was found with Sb-1 and rifampin combination against MRSA or MSSA (**Supplementary Table S2**).

DISCUSSION

Biofilm formation on the device surface is the key occurrence in the pathogenesis of implant-associated infections, requiring the use of biofilm-active antibiotics (Davidson et al., 2019). Rifampin emerged about three decades ago as an antibiofilm antibiotic

TABLE 3 | Antibiofilm effects of simultaneous ($MBEC_{SIM}$) or staggered ($MBEC_{STA}$) phage–antibiotic combinations against clinical strains.

Antibiotic	**VAN**		**DAP**		**FOF**		**GEN**	
MRSA strains	**$MBEC_{SIM}$**	**$MBEC_{STA}$**	**$MBEC_{SIM}$**	**$MBEC_{STA}$**	**$MBEC_{SIM}$**	**$MBEC_{STA}$**	**$MBEC_{SIM}$**	**$MBEC_{STA}$**
MRSA1	>256 (NS)[a]	>256 (NS)[a]	8 (0.125, S)	8 (0.125, S)	>256 (NS)[a]	64 (0.06, S)[b]	64 (0.25, S)	64 (0.25, S)
MRSA2	>256 (NS)[a]	>256 (NS)[a]	16 (0.125, S)	16 (0.125, S)	>256 (NS)[a]	256 (0.25, S)[b]	256 (0.5, NS)	64 (0.125, S)
MRSA3	>256 (NS)[a]	>256 (NS)[a]	16 (0.25, S)	16 (0.25, S)	>256 (NS)[a]	>256 (NS)[a]	128 (0.25, S)	64 (0.125, S)
MRSA4	>256 (NS)[a]	>256 (NS)[a]	16 (0.125, S)	16 (0.125, S)	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]
Antibiotic	**FLU**		**CFZ**		**FOF**		**GEN**	
MSSA strains	**$MBEC_{SIM}$**	**$MBEC_{STA}$**	**$MBEC_{SIM}$**	**$MBEC_{STA}$**	**$MBEC_{SIM}$**	**$MBEC_{STA}$**	**$MBEC_{SIM}$**	**$MBEC_{STA}$**
MSSA1	128 (0.125, S)	64 (0.006, S)	>256 (NS)[a]	256 (0.25, S)[b]	>256 (NS)[a]	>256 (NS)[a]	128 (0.25, S)	128 (0.25, S)
MSSA2	256 (0.25, S)	32 (0.003, S)	>256 (NS)[a]	128 (0.125, S)[b]	>256 (NS)[a]	64 (0.006, S)[b]	128 (0.25, S)	128 (0.25, S)
MSSA3	256 (0.25, S)	32 (0.003, S)	>256 (NS)[a]	256 (0.25, S)[b]	>256 (NS)[a]	256 (0.25, S)[b]	>256 (NS)[a]	>256 (NS)[a]
MSSA4	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]	256 (0.25, S)[b]	>256 (NS)[a]	>256 (NS)[a]
MSSA5	256 (0.5, NS)	256 (0.5, NS)	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]	>256 (NS)[a]
MSSA6	>256 (NS)[a]	32 (0.003, S)[b]	>256 (NS)[a]	16 (0.015, S)[b]	>256 (NS)[a]	32 (0.003, S)[b]	256 (0.25, S)	256 (0.25, S)

MBEC concentration values are expressed in μg/ml. In brackets is shown the ratio value followed by the ratio interpretation. VAN, vancomycin; FLU, flucloxacillin; DAP, daptomycin; CFZ, cefazolin; FOF, fosfomycin; GEN, gentamicin; S, synergism; NS, no synergism. [a]MBEC value above 1/4x$MBEC_{alone}$ (considering $MBEC_{alone}$ equal to 1,024 μg/ml), thus $MBEC_{phage}/MBEC_{alone}$ ratio is interpreted as >0.25 (NS). [b]$MBEC_{alone}$ considered equal to 1,024 μg/ml for $MBEC_{phage}/MBEC_{alone}$ ratio calculations.

against *S. aureus* orthopedic implant-associated infections (Zimmerli and Sendi, 2019), presenting good penetration and bioavailability in osteo-articular tissue (Sendi and Zimmerli, 2017). In this study, we investigated alternatives to rifampin for the treatment of implant-associated infections caused by RRSA.

Phage efficacy has been described to be influenced by host specificity, among several other factors (Ly-Chatain, 2014). In our study, Sb-1 showed high killing effect against most tested strains, but still a complete biofilm eradication with the phage alone was not achieved, possibly due to the establishment of an equilibrium between virus and host, as reported earlier (Głowacka-Rutkowska et al., 2019), what might be prevented with the addition of antibiotics.

The phage–antibiotic combinations tested in our study were selected based on the methicillin-resistant profile of the *S. aureus* isolates, as usually done in the clinical setting (Berbari et al., 2020). Hence, in addition to testing fosfomycin and gentamicin against all strains, daptomycin and vancomycin were selected for testing on MRSA strains, while flucloxacillin and cefazolin were selected for testing on MSSA strains. For the evaluation of phage–antibiotic combinations, a fixed value of 1,024 μg/ml was considered for the calculation of the $MBEC_{phage}/MBEC_{alone}$ ratios for samples with $MBEC_{alone}$ > 1,024 μg/ml. It should be noted that, by this approach, some combinations that were interpreted as not synergistic could turn out to have a synergistic effect when testing higher MBEC values. However, the observed positive synergistic effects of phage–antibiotic combinations with our experimental setup are certain and usually presenting considerably lower MBEC values compared to the MBEC values of single antibiotics.

The determination of the EOP ratios is a frequent test to identify phages suitable for phage therapy (Khan Mirzaei and Nilsson, 2015). In our study, however, we did not observe a correlation between the EOP rank and the antibiofilm activity of the phage against a specific strain. For instance, Sb-1 showed low efficacy against MSSA ATCC 29213 biofilm with no synergistic effect in combination with antibiotics despite a high EOP rank, but Sb-1 in combination with daptomycin resulted in a synergistic effect against MRSA3 and MRSA4 although showing lower EOP ratios on these strains. Thus, in the context of using phages to control bacterial biofilms, the determination of the EOP ratios should not be misinterpreted toward a correlation to efficiency against biofilms. The nature of the biofilm matrix can differ among strains, ultimately affecting the bioavailability and/or function of an antimicrobial, as suggested by Bauer and coworkers (Bauer et al., 2013), who evaluated antibiotic activity on young and mature MSSA and MRSA biofilms and observed that, besides biofilm maturity, the bacterial strain clearly influenced antibiotic activity.

The order of administration when combining antibiotics and phages has been shown to play a key role for a synergistic antimicrobial effect (Dickey and Perrot, 2018; Kumaran et al., 2018). We observed a synergistic effect when Sb-1 was combined with fosfomycin or cefazolin by staggered application but not when these antibiotics and Sb-1 were applied simultaneously. Moreover, the pre-exposure to Sb-1 followed by flucoxacillin eradicated the biofilm at lower antibiotic concentrations compared to simultaneous application. These findings indeed seem to indicate that exposure of biofilms first to phage followed by antibiotics is the most effective way to eradicate them. Previous studies have shown the benefit of the staggered application when combining antibiotics and phages (Tkhilaishvili et al., 2018), while a simultaneous exposure could result in hindering their antibiofilm efficacy, possibly due to antagonistic modes of action (e.g., antibiotics interfering with the bacterial DNA replication process) or due to the killing of host bacteria – which is essential for phage production – by the antibiotic (Chaudhry et al., 2017; Kumaran et al., 2018; Akturk et al., 2019).

On the other hand, other than with the MRSA ATCC strain, combining Sb-1 and daptomycin or vancomycin against rifampin-resistant MRSA strains exhibited the same outcome independently of the order of administration. In a previous study by Dickey and Perrot (2018), the authors also showed that a simultaneous treatment of *S. aureus* biofilm with daptomycin and phage was as effective as sequential treatment. Moreover, they showed an antagonistic effect when combining phage and vancomycin. Considering that the wall teichoic acid from the bacterial cell wall is the primary staphylococcal phage receptor (Azam and Tanji, 2019) and that vancomycin has a unique mechanism of action inhibiting cell wall synthesis (Watanakunakorn, 1984), it is conceivable that phage infection was negatively affected by the vancomycin impact on the bacterial cell wall. Daptomycin action disrupting the bacterial cell membrane structure seems to have a lower interference with phage action. As shown also by Dickey and Perrot, the simultaneous application of phage and daptomycin at 10xMIC allowed phage growth, whereas most antibiotics tested in their study at 10xMIC either prevented phage growth (ciprofloxacin, vancomycin, and tetracyclin) or led to massive decreases in phage density (gentamicin, erythromycin, and linezolid) (Dickey and Perrot, 2018).

Generally, vancomycin is recommended for the treatment of MRSA implant-associated infections (Paiva and Eggimann, 2017), yet a high rate of vancomycin treatment failure in vancomycin-susceptible MRSA infections has been reported (Dombrowski and Winston, 2008; Abdelhady et al., 2013). This observation correlates with our findings showing the inefficiency of treating biofilms of clinical strains with vancomycin alone or combined with Sb-1. The low efficacy of vancomycin against staphylococcal biofilms could be due to a reduced biofilm penetration, a reduced concentration of free vancomycin being sequestrated by *S. aureus* on peptidoglycan layers, or a stimulation of biofilm formation by low concentrations of vancomycin (Broussou et al., 2018). Conversely, daptomycin has shown a superior efficacy against bone and joint infections caused by MRSA (Chang et al., 2017; Telles et al., 2019). The consistent synergistic effect observed with Sb-1/daptomycin combination against all tested rifampin-resistant MRSA strains in our study, together with the good biofilm penetration properties (Ozturk et al., 2016) and *in vitro* activity against stationary-phase bacteria inside the biofilm (Smith et al., 2009), makes daptomycin a promising candidate for combinatorial therapy.

Another promising therapeutic approach based on our results was found by the combination of Sb-1 with flucloxacillin for the eradication of rifampin-resistant MSSA strains, where there was a remarkable reduction in the MBEC values, and $MBEC_{phage}/MBEC_{alone}$ ratios as low as 0.003, could be observed after staggered phage-antibiotic administration against 67% of the strains. Analysis on the production of type A beta-lactamase by MSSA strains, responsible for cefazolin hydrolysis (Nannini et al., 2009), could bring insights on the lower antibiofilm efficacy of cefazolin compared to flucloxacillin.

Low $MBEC_{phage}/MBEC_{alone}$ ratios (ranging from 0.003 to 0.06) were also observed with staggered administration of Sb-1 with fosfomycin. Fosfomycin has been shown to act synergistically with other antibiotics against biofilms of different bacterial species, including MRSA, in part probably because of its broad-spectrum bactericidal activity (Chai et al., 2016; Wang L. et al., 2019). In addition, favorable characteristics associated to fosfomycin include the ability to break up biofilms and enhance the permeability of other antimicrobials and a presumed immunomodulatory effect (Mihailescu et al., 2014).

Previous studies revealed synergistic effects by combining phage with an antibiotic for which the bacteria strain was resistant (Liu et al., 2020). In our study, however, the use of Sb-1 in combination with rifampin against biofilms of RRSA strains, as well as Sb-1/fosfomycin and Sb-1/gentamicin combinations against MRSA3 and MRSA ATCC 43300, respectively, did not reveal an improved anti-biofilm effect.

When trying to draw conclusions or make clinical extrapolations, it is important to consider the small number of tested strains in our study. We aimed to provide the first original data on the combinatorial effect of Sb-1 and different antibiotics to eradicate RRSA biofilms *in vitro*. Our work highlights that findings obtained testing ATCC strains may differ from the outcome with clinical isolates, but also among the different strains, implying that selecting an appropriate phage–antibiotic combinatorial therapy will be highly dependent on the strain causing the infection as well as on the specific antibiofilm efficacy of the phage, more than its lytic spectrum. Moreover, there is substantial evidence that many antibiotics can interfere with phage infection activity—especially at concentrations exceeding measured minimum inhibitory concentrations—and thus with phage primary pharmacodynamic properties. Hence, further preclinical and clinical studies are essential to support the development of phage/antibiotic combination therapy with particular isolates. Factors that point toward a more personalized approach for the successful treatment of antibiotic-resistant implant-associated infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LW and TT performed the experiments with the contribution of MG. MG, LW, and TT analyzed the data. MG and TT drafted the manuscript with the contribution of LW and AT. All authors conceived and designed the experiments and revised and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.602057/full#supplementary-material

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Eco-Evolutionary Effects of Bacterial Cooperation on Phage Therapy: An Unknown Risk?

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If there is something we have learned from the antibiotic era, it is that indiscriminate use of a therapeutic agent without a clear understanding of its long-term evolutionary impact can have enormous health repercussions. This knowledge is particularly relevant when the therapeutic agents are remarkably adaptable and diverse biological entities capable of a plethora of interactions, most of which remain largely unexplored. Although phage therapy (PT) undoubtedly holds the potential to save lives, its current efficacy in case studies recalls the golden era of antibiotics, when these compounds were highly effective and the possibility of them becoming ineffective seemed remote. Safe PT schemes depend on our understanding of how phages interact with, and evolve in, highly complex environments. Here, we summarize and review emerging evidence in a commonly overlooked theme in PT: bacteria-phage interactions. In particular, we discuss the influence of quorum sensing (QS) on phage susceptibility, the consequent role of phages in modulating bacterial cooperation, and the potential implications of this relationship in PT, including how we can use this knowledge to inform PT strategies. We highlight that the influence of QS on phage susceptibility seems to be widespread but can have contrasting outcomes depending on the bacterial host, underscoring the need to thoroughly characterize this link in various bacterial models. Furthermore, we encourage researchers to exploit competition experiments, experimental evolution, and mathematical modeling to explore this relationship further in relevant infection models. Finally, we emphasize that long-term PT success requires research on phage ecology and evolution to inform the design of optimal therapeutic schemes.

Keywords: phages, phage therapy, quorum sensing, bacteria-phage interactions, cheaters, anti-phage defense

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INTRODUCTION

In some bacteria, such as *Vibrio cholerae*, phage defense mechanisms are induced by quorum sensing (QS), whereas in others, such as *Pseudomonas aeruginosa*, QS promotes phage susceptibility. Whether bacteria use QS to repress or promote virulence, increasing evidence points to a close connection between QS and modulation of susceptibility to phage infection. The perspective presented here aims to briefly revisit phage therapy (PT) in view of recently reported

phage-host interactions, summarize emerging evidence on the QS-phage susceptibility relationship, discuss potential implications in the context of PT, and explore how this knowledge may help to develop antibacterial strategies that reduce the risk of undesired side effects.

REVISITING PHAGE THERAPY

The idea of using bacteriophages to treat bacterial infections, namely, PT, is as old as phage discovery itself, however, the introduction and widespread use of antibiotics, among other factors, limited further PT development on a global scale (Casey et al., 2018). Given the current antibiotic crisis, PT is facing a prominent second wave of interest supported by successful case reports (Sarker et al., 2016; Chan et al., 2018; Duplessis, 2018; Furr et al., 2018; LaVergne et al., 2018; Dedrick, 2019) and creation of PT-oriented research centers and private biotech companies (Adaptive-Phage-Therapeutics, n.d.; Center for Innovative Phage Applications and Therapeutics, n.d.; PhagoMed, n.d.). This year, the use of bacteriophages was proposed as a potential "game changer" in the efforts to reduce the mortality rate in patients infected with the SARS-Cov-2 virus, and particularly in those developing secondary bacterial infections (Wojewodzic, 2020), whereas other research work reported the potential use of phage-derived enzymes in controlling the emergence of intestinal pathobionts (Fujimoto et al., 2020). Notably, these reports reflect the current enthusiasm for applying PT on a large scale and in a broad variety of health problems.

As PT advances toward becoming a true medical alternative, questions naturally arise about potential limitations and negative effects of using phages as antimicrobials. Determinants limiting phage infection (e.g. spatial structure of the infection environment and microbial community diversity), potential incompatibility with other therapies, difficulties on the production and stability of phage stocks, safety and side effects issues, represent factors impeding further PT development (Oechslin, 2018; Torres-Barceló, 2018; Breederveld, 2019). Importantly, several of these factors can be linked to our lack of knowledge about the ecological and evolutionary impact of PT in an ecosystem consisting not only of the therapeutic phage and its target pathogen but also the host microbiome and immune system.

The coexistence of multiple biotic and abiotic elements at the site of infection implies a complex network with a plethora of interactions about which very little is currently known. The effect of phages on the immune system and selection of resistance to phage infection stand as some of the major PT concerns resulting from this interaction network. As recent research shows, phage interaction with the immune system is intricate and can subvert immune response (Sweere et al., 2019). Filamentous phages infecting *P. aeruginosa* can physically interact with mammalian immune cells triggering a maladaptive immune response that results in impaired bacterial clearance during infections (Sweere et al., 2019). These phages have been also involved in an immune response impairing keratinocyte migration and leading to delayed healing of *P. aeruginosa*-infected wounds (Bach et al., 2020). Although filamentous phages do not represent a virus type selected for therapy purposes, these studies highlight the complex consequences of previously unknown interactions between phages and the immune system. Furthermore, this interaction is not restricted to filamentous phages, since viruses of the order Caudovirales, typically selected for PT, can also directly interact with immune systems, stimulating a response that exacerbates intestinal inflammation and colitis (Bollyky, 2019; Gogokhia et al., 2019).

In recent years, progress has also been made on the understanding of how bacteria evolve cross-resistance against multiple phages. For example, the comprehensive characterization of a bacteria-phage interaction network indicated that cross-resistance is common and associated with different genetic basis related to exposure to distinct phages (Wright et al., 2018). Yet, the study showed that mutations in diverse phage receptors structure the modularity of the network and feature lower fitness cost than other mutations in regulatory genes driving more generalist phage resistance (Wright et al., 2018). Further research revealed that timing and order of phage exposure are factors that shape the evolutionary trajectory of cross-resistance as they are associated with a different mutational basis, strength of resistance, and fitness cost (Wright et al., 2019). These studies underscore the intricate nature of phage-bacteria interactions and their impact in the emergence of key evolutionary traits such as phage resistance, a factor that should be considered in the design of long-term PT strategies.

We seem to be on the brink of using PT on a larger scale, therefore, it is pivotal to start addressing how the interaction of phages with elements of the surrounding environment, especially in the context of infections, can impact PT outcomes. Here, we focus on the implications of a so far overlooked interaction with a profound effect in the biology of bacteria and their viruses: phages and cell-cell communication or QS. Emerging evidence reveals that this interrelationship is very complex and multidimensional since QS can regulate various anti-phage defense mechanisms, whereas phages can influence cooperative behaviors and hack QS systems to mediate their own gene expression. Phage-associated intercellular communication will not be discussed in this perspective but it has been recently addressed by Igler and Abedon (2019).

QUORUM SENSING, COOPERATION, AND CHEATING

QS is a prevalent mechanism for gene expression regulation in bacterial populations *via* self-produced and diffusible signal molecules (Schuster et al., 2017). QS primarily coordinates cooperative behavior (e.g., virulence and nutrient digestion) through the secretion of extracellular products such as toxins, exopolysaccharides, or enzymes (Schuster et al., 2017). These secreted products are considered public goods since the population benefits from them in a cell density-dependent manner. Importantly, besides public goods, QS can control the production of traits that only benefit the producer individual, hence referred to as private goods (Schuster et al., 2017).

QS-mediated cooperation is a key population attribute; however, its maintenance is often challenged by the emergence

of individuals that profit from public goods without contributing to their production, mutants commonly known as cheaters. Exploitation by cheaters can ultimately lead to population collapse but several factors preventing this scenario have been reported, including the action of QS-controlled private goods that stabilize cooperation (Dandekar et al., 2012; García-Contreras et al., 2014). In this context, preferential phage infection toward a subpopulation, namely producers or cheaters, typically represented by wild-type or mutant individuals, can define the ecological and evolutionary fate of the population. This situation may occur in infections where the emergence of QS mutants has been documented. *P. aeruginosa* (Hoffman et al., 2009; Wang et al., 2018) and *Staphylococcus aureus* (Paulander et al., 2012) represent examples of pathogens for which the occurrence of QS mutants has been reported in the clinic. Therefore, investigating the effect of QS on phage susceptibility is relevant to anticipate PT outcomes, especially considering that QS is commonly involved in virulence regulation and selecting one subpopulation could derive unintended consequences.

QUORUM SENSING AND ANTI-PHAGE DEFENSE MECHANISMS

A growing body of evidence implicates QS in regulating anti-phage defense strategies. In some instances, QS coordinates downregulation of diverse cell membrane proteins acting as phage receptors, thus resulting in reduced phage susceptibility. This is the case for the receptor LamB in *Escherichia coli* (Høyland-Kroghsbo, 2013), OmpK in *Vibrio anguillarum* (Tan et al., 2015), and O1 in *V. cholerae* (Hoque, 2016). In *E. coli*, QS controls the concomitant downregulation of multiple receptors, including the flagellum, thus widening the range of protection against phage infection. Likewise, in *V. cholerae*, QS is additionally associated with increased emergence of phage resistant individuals and production of hemagglutinin protease, a protein causing extracellular phage inactivation (Hoque, 2016). More recently, QS was also linked to promotion of extracellular proteolytic activity affecting the virion stability of different phages in *V. anguillarum* (Tan et al., 2020). In this study, the authors also report the association between QS and reduction of H20-like prophage induction (Tan et al., 2020), adding another layer of complexity to the phage-bacteria interactions *via* density-dependent cell-cell communication.

P. aeruginosa represents another example where QS has been associated with reduction in plaque production, burst size, and expression of the phage RNA polymerase (Qin et al., 2017). Although the mechanism behind this response has yet to be deciphered, it seems to be independent of phage adsorption and related to an increase in the proportion of dormant cells (Qin et al., 2017). It is worth noting, however, that this response appears to be strain-dependent, as other studies in *P. aeruginosa* show a different QS-phage infection relationship (see below). Activation of the adaptive immune system CRISPR-Cas has been also associated with QS (Høyland-Kroghsbo et al., 2017), however, it is unclear to what extent a deficient QS system would impact phage susceptibility *via* this mechanism.

The link between QS and anti-phage defense is consistent with the increased risk in spread of infection at higher cell densities, but also implies a strong role for phages in mediating intra-population bacterial competition. In most of the cases described thus far, QS-mediated defense mechanisms provide a private benefit to the producers' subpopulation (e.g., lower receptor expression or metabolic activity), but at least two examples correspond to anti-phage protection as a public good (hemagglutinin protease production and promotion of extracellular proteolytic activity). In the context of PT, the theory suggests that QS-controlled phage tolerance as a private good could lead to selection of the producer's subpopulation, in view of their lower susceptibility to phage infection compared to the cheater individuals. On the other hand, phage defense in the form of a public good portrays a more complex scenario because it would be prone to cheating, as we discuss below.

Remarkably, the QS-phage infection interlink is not unidirectional, since QS has also been connected with increased phage susceptibility. In *E. coli* (Taj et al., 2014) and *P. aeruginosa* (Glessner et al., 1999; Mumford and Friman, 2017; Saucedo-Mora et al., 2017), QS has been linked with higher infection rate, represented by expanded plaque production, plaque size, or overall lytic activity. A role of QS in the assembly of type 4 pili, a phage receptor involved in cell twitching motility, was hypothesized to be the cause of increased phage susceptibility in *P. aeruginosa* (Glessner et al., 1999). Although further research uncoupled the direct link between QS and twitching motility, the study revealed that QS mutants readily accumulate secondary mutations associated with loss of twitching motility (Beatson et al., 2002). Other mechanisms underlying the QS-promoted phage susceptibility in *P. aeruginosa* and *E. coli* remain largely unknown. This alternative side of the relationship between QS and phage susceptibility also has potential implications in the PT context: in principle, phages could infect producer individuals preferentially, ultimately selecting the cheaters subpopulation over time. Such selection could have positive consequences in terms of virulence in pathogens where this trait is controlled by QS; nevertheless, other factors may help to mitigate the strength of selection given the importance of QS in regulating multiple cooperative attributes in the population. In **Table 1**, we compile examples of QS-Phage interactions and briefly describe hypothetical scenarios about their impact on intrapopulation competition and PT outcomes.

DISCUSSION

It is becoming increasingly clear that, among the broad array of bacteria-phage interactions, the link between QS and phages has a profound impact in the eco-evolutionary dynamics of microbial communities, therefore, it represents a relevant factor to consider in the context of infections and PT.

QS is a key topic in bacterial infection research since it regulates the production of virulence factors in several pathogens (Rutherford and Bassler, 2012; Castillo-Juárez et al., 2015). As these factors are typically used by the population to collectively change or take advantage of the surrounding environment, they are considered

TABLE 1 | Influence of quorum sensing on phage susceptibility

QS reduces phage susceptibility					
Phage	**Type**	**Host**	**Reference**	**Potential effect in intra-population competition**	**Hypothetical impact in PT**
KVP40	Virulent	*V. anguillarum*	Tan et al., 2015	In a mixture of wild-type and QS-deficient bacteria, the phage will infect the mutant phenotype preferentially as a result of the higher phage receptor expression in the QS-deficient cells, thus resulting in selection of the wild-type phenotype over time.	
Various (15)	Virulent	*V. cholerae*	Hoque, 2016		
Lambda, X	Temperate, Unknown	*E. coli*	Høyland-Kroghsbo, 2013		
K5, C11	Virulent	*P. aeruginosa*	Qin et al., 2017	In a mixture of wild-type and QS-deficient bacteria, the phage will be more successful at infecting the mutant individuals due to the lower metabolic activity of the wild-type strain, thus promoting the selection of the wild-type phenotype over time.	In cases where QS positively regulates bacterial virulence factors, PT could lead to increased virulence: if phages fail to eradicate the target bacterial population, the surviving individuals will most likely correspond to virulent wild-type cells that can then proliferate at the site of infection.
QS increases phage susceptibility					
Phage	**Type**	**Bacterium**	**Reference**	**Potential effect in intra-population competition**	**Hypothetical impact in PT**
D3112cts	Temperate	*P. aeruginosa*	Glessner et al., 1999	Phage will attack the wild-type population at a higher rate due to higher expression of the phage receptor	If PT fails to eradicate bacteria, it will potentially select a sub-population with low virulence and prone to be eliminated by the immune system.
D3112, JBD30	Temperate	*P. aeruginosa*	Saucedo-Mora et al., 2017	Phages infect the wild-type strain more efficiently, however, in a mixture of wild-type and QS-deficient bacteria, phages select the wild-type phenotype, likely resulting from more efficient lysogenesis in the wild-type population leading to fitness increase	If PT fails to eradicate bacteria, it will potentially select the wild-type phenotype, increasing virulence
T4	Virulent	*E. coli*	Taj et al., 2014	In a mixture of wild-type cells and mutants unable to detect QS signals, the phage will exhibit higher lytic activity in wild-type individuals if acyl-homoserine lactone signaling molecules are present in the medium. In contrast, in presence of the indole signal, the phage will reduce its lytic activity in cells able to detect this molecule. Therefore, selection will depend on both the availability of signal molecules and the ability of the cells to sense them.	The effectiveness of PT, resulting from the preferential eradication of the wild-type or mutant sub-population, will be determined by the type of signaling molecules in the surrounding environment.

Results from the work by Mumford and Friman (2017) were omitted in this table because the experiments involved inter-species bacterial competition in addition to the different QS genotypes, hence, predicting the particular effect of QS in selection becomes more complex; nevertheless, we highlight the relevance of their findings, especially in the context of PT.

public goods. Cooperative behaviors require that many cells contribute to the pool of public goods to achieve efficiency. Nonetheless, this concerted effort is commonly prone to cheating by individuals that benefit from the pool of goods without contributing to it. The emergence of cheaters represents an interesting phenomenon in the context of infections. *P. aeruginosa* and *S. aureus* are examples of pathogens that use QS to control the expression of several virulence genes and for which mutations in QS genes (e.g., *lasR/rhlR* or *agr* genes, respectively) occur in infections (Hoffman et al., 2009; Paulander et al., 2012; Wang et al., 2018). The implications of this evolutionary process are unclear but this observation shows that QS mutants and isolates with wild-type QS genes occur during the course of infection.

Several QS-phage interactions reported so far show that cell-cell communication enhances bacterial survival against phages. Due to its nature, this anti-phage strategy confers enhanced phage tolerance, i.e., transient reduction in susceptibility to infection. The fact that similar relationships can be found in different bacterial species suggests, as other authors point out (Høyland-Kroghsbo, 2013; Høyland-Kroghsbo et al., 2017; Qin et al., 2017; Saucedo-Mora et al., 2017), that this anti-phage defense system is widely distributed. The phage defense attribute typically corresponds to a private good in the form of downregulation of the phage receptor, therefore, it can be expected that other mechanisms protecting the cell surface from interaction with phages can be regulated by QS as well.

Importantly, the coupling of public goods production with private phage defense under the same regulation mechanism, QS, implies that phages can be key factors in stabilizing cooperation in mixed populations. In this type of interaction, the theory suggests preferential infection of QS mutants, which could have important implications in the context of PT if the target pathogen upregulates virulence genes through QS: selection of QS-proficient cells could cause an increase in virulence, either transient or stable depending on the efficacy of the defense system and hence the strength of the selection. Nevertheless, this interaction also opens the possibility of the combined use of PT and quorum quenching (QQ), which would increase the phage susceptibility of the population while decreasing virulence. As previously highlighted (Hoque, 2016), it is important to characterize the effect of QQ in the target pathogen to assess compatibility with PT, as in some cases, such as *V. cholerae*, QQ may promote increased virulence.

There are documented examples where QS-mediated phage defense represents a public good: proteases production by *V. cholera* (Hoque, 2016) and *V. anguillarum* (Tan et al., 2020). These extracellular enzymes have been proven to inactivate phage virions, potentially protecting both producer and cheater individuals in mixed populations. It is worth noting that predicting the competition outcome in an infection scenario becomes more difficult when considering the additional effect of public goods, as the final subpopulations proportion after PT treatment would depend on complex interactions; e.g. the strength of positive selection on the wild-type phenotype by lowering phage receptor expression and inactivating phages *via* proteases, combined with a negative selection factor resulting from the protease-mediated protection of QS mutants. This type of interactions, however, is particularly suitable to be investigated through experimental evolution and mathematical modeling approaches to simulate a series of scenarios (see Pérez-Velázquez et al., 2016; Cazares et al., 2020).

Intriguingly, the QS-phage interaction can have contrasting outcomes in terms of susceptibility to infection. Reports in *P. aeruginosa* and *E. coli* show that QS can promote phage susceptibility, although the mechanisms behind this effect are still unknown. This interaction type suggests that phages primarily target producer individuals, which could lead to selection of QS mutants. In a hypothetical infection scenario, this selection may decrease virulence if it is QS-regulated, and QQ may counteract PT since QS disruption would lead to reduction in phage susceptibility. It should be noted, however, that given the relevance of active QS systems in bacteria, other factors can play a role in stabilizing cooperation in the population, hence offsetting the selection pressure exerted by phages. One example is our work on selection of functional QS systems by *P. aeruginosa* temperate phages (Saucedo-Mora et al., 2017). We showed that D3112-like phages promote the relative fitness of QS-active cells compared to the signal blind *lasR rhlR* double mutants in mixed planktonic populations, thus counteracting exploitation by cheaters and selecting functional QS systems. In our study, phages replicated preferentially in the wild-type strain, predicting selection of the mutant population, however, we observed the opposite effect. We hypothesize that lysogeny was a key factor in the selection: since phages infect the wild-type isolates preferentially, the population of QS-proficient lysogens that become resistant to phage infection by homoimmunity grows rapidly, overtaking the cheaters subpopulation. The role of temperate phages in increasing competitiveness has been documented in *P. aeruginosa* (Davies et al., 2016). Additionally, there are multiple reports of QS-controlled factors (e.g., pyocyanin and rhamnolipids) promoting negative selection of the cheater's subpopulation in this bacterial species (Wang et al., 2015; Castañeda-Tamez et al., 2018; García-Contreras et al., 2020).

It is worth noting that the findings on QS-phage interactions described above correspond to reports on both virulent and temperate phages (see **Table 1**, column 2). Historically, virulent phages have been preferred for therapy purposes over temperate viruses, mostly due to concerns on the latter potential to transfer virulence determinants and drive lysogenic conversion (Monteiro et al., 2018). This suggests that we should focus on the interactions with virulent phages, yet the QS-controlled anti-phage mechanisms reported so far seem to be generalist (e.g. downregulation of phage receptors and production of virion-inactivating extracellular proteases), and hence relevant to both phage types. Further research expanding susceptibility testing to a wider panel of phages and characterizing new anti-phage strategies will be necessary to confirm that this is the case. Moreover, recent advances in the fields of genomics and synthetic biology have led to a renewed interest in exploring the potential of temperate phage variants as therapeutic agents (see the review by Monteiro et al., 2018), underscoring the importance of using a broad variety of phages to characterize QS-driven shifts in susceptibility to infection.

New research shows that filamentous phages may represent public goods themselves, potentially affecting the sociobiology of their hosts. Apart from their role in immune system modulation, these phages of the *Inoviridae* family can promote virulence by enhancing the properties of biofilms toward increased adherence, and tolerance to antibiotics and desiccation (Secor et al., 2015). Future research will reveal whether strategies allowing cheating of filamentous phages exist, as expected from the cooperative nature of their production, and what bacterial mechanisms regulate the synthesis of this viral group.

The reports compiled here underline the wealth and diversity of bacteria-phage interactions, and their potential to affect the sociobiology of microbial populations, including in bacterial infections. The perspective presented is not an isolated effort, since the relevance of phage social interactions has been recently reviewed by Fernández et al. (2018) and Secor and Dandekar (2020). In particular, we underscore the importance of understanding the interplay between phages and bacterial communication strategies. It is clear that more research is necessary to assess how this relationship can impact PT efforts. For example, phage defense as a private good under the control of QS implies that phages help stabilizing cooperation, however, for this link to be evolutionary stable it should have a direct fitness benefit dependent on cell density (Schuster et al., 2017). This effect should then be tested through competition experiments in different conditions, especially in infection models, and using experimental evolution approaches. Not only the therapeutic phage and its target pathogen will exist in an infection, therefore, the fate of these populations will be shaped by their interaction with the surrounding environment,

including the microbial community. Remarkably, elimination of a strain in the ecosystem could lead to the unintended selection of a different pathogen. In this regard, research shows that both QS and inter-species bacterial competition can indeed affect the outcome of phage infection in an *in vitro* PT model (Mumford and Friman, 2017). Furthermore, the QS-phage infection interlink can derive in distinct results depending on the bacterial pathogen, hence, a diversity of models needs to be investigated to make robust conclusions about this relationship. Finally, mathematical modeling can provide valuable insights into the outcome of complex interactions between multiple factors but it requires prior experimental knowledge of the system (Cazares et al., 2020); thus, we encourage others to integrate both approaches in any phage infection system aiming to recapitulate PT.

In concordance with others (see references in **Table 1**; Fernández et al., 2018; Secor and Dandekar, 2020), we highlight that discovery of phage interactions with various systems and discussion of their implications can be used to advance phage-based therapies. The study of phage-immune system interactions can lead to the development of vaccines protecting against bacterial infections (Sweere et al., 2019). Investigation of bacteria-phage interaction networks can be used to inform selection of phages, conditions, and order of application to optimize therapy (Wright et al., 2019). Likewise, our knowledge on QS-phage interactions and their role in bacterial cooperation can inform the combined use of PT and QQ, and aid in the selection of phages that are not affected by QS-driven defense mechanisms or that preferentially infect cells featuring QS-controlled virulent phenotypes. As PT steadily advances toward a systematic use in clinical environments, we emphasize that research in phage ecology and evolution is not desirable but critical for PT success, as it can support the design of strategies that minimize the risk of unintended side effects and optimize the stability and efficacy of therapy.

AUTHOR CONTRIBUTIONS

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

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Genomic, Morphological and Functional Characterization of Virulent Bacteriophage IME-JL8 Targeting *Citrobacter freundii*

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Citrobacter freundii refers to a fish pathogen extensively reported to be able to cause injury and high mortality. Phage therapy is considered a process to alternatively control bacterial infections and contaminations. In the present study, the isolation of a virulent bacteriophage IME-JL8 isolated from sewage was presented, and such bacteriophage was characterized to be able to infect *Citrobacter freundii* specifically. Phage IME-JL8 has been classified as the member of the *Siphoviridae* family, which exhibits the latent period of 30–40 min. The pH and thermal stability of phage IME-JL8 demonstrated that this bacteriophage achieved a pH range of 4–10 as well as a temperature range of 4, 25, and 37°C. As revealed from the results of whole genomic sequence analysis, IME-JL8 covers a double-stranded genome of 49,838 bp (exhibiting 47.96% G+C content), with 80 putative coding sequences contained. No bacterial virulence- or lysogenesis-related ORF was identified in the IME-JL8 genome, so it could be applicable to phage therapy. As indicated by the *in vitro* experiments, phage IME-JL8 is capable of effectively removing bacteria (the colony count decreased by 6.8 log units at 20 min), and biofilm can be formed in 24 h. According to the *in vivo* experiments, administrating IME-JL8 (1×10^7 PFU) was demonstrated to effectively protect the fish exhibiting a double median lethal dose (2×10^9 CFU/carp). Moreover, the phage treatment led to the decline of pro-inflammatory cytokines in carp with lethal infections. IME-JL8 was reported to induce efficient lysis of *Citrobacter freundii* both *in vitro* and *in vivo*, thereby demonstrating its potential as an alternative treatment strategy for infections attributed to *Citrobacter freundii.*

Keywords: *Citrobacter freundii*, bacteriophage, phage therapy, biofilm, phage genome

INTRODUCTION

Citrobacter freundii (*C. freundii*) refers to a facultative anaerobic Gram-negative bacillus that has been reported to exist in soil, water, food and the natural environment (e.g., hospitals). It pertains to the family *Enterobacteriaceae*. *C. freundii* belongs to a normal flora of the fish and human intestine, as well as a conditionally pathogenic bacteria capable of infecting people that exhibit low immunity, which will cause several diseases (e.g., pneumonia, meningitis, sepsis, bacteremia, and urinary tract infections) (Anderson et al., 2018; Ando et al., 2018; Liu et al., 2018). Furthermore, *C. freundii* has been associated with numerous diseases and symptoms (e.g., hemorrhagic septicemia, severe enteritis and serious lesions in kidney and gills of catfish; systemic infection in common carp; tail necrosis, septicemia, hemorrhage, as well as reddening of the body in Mozambique tilapia; high mortality in Nile tilapia; cutaneous hemorrhages in zebrafish; tail necrosis, septicemia, hemorrhage as well as reddening of the body in Mozambique tilapia; high mortality in Nile tilapia; gastroenteritis and progressive high-mortality in rainbow trout; systemic infection in common carp) (Zurfluh et al., 2017; Castanheira et al., 2018; Hassen et al., 2020). *C. freundii* has been reported to resist numerous commonly used antibiotics since its discovery. Over the past few years, as impacted by the indiscriminate use of antibiotics and weak supervision of drug-resistant bacteria, a growing number of multi-drug resistant *C. freundii* were clinically detected (Ahad et al., 2017). *C. freundii* can form surface-related complex communities, which was considered biofilms in both food matrices and hospital settings. However, it can resist dehydration, UV radiation, common chemical sanitizers, as well as detergents. As a matter of fact, antimicrobial agents used routinely can inhibit its growth only, while no such treatments have been found effective to remove *C. freundii* in hospital environments (Santos et al., 2017). Phage treatment was described as a promising approach to regulate pathogens and reduce biofilms.

Bacteriophage or phage refer to a type of virus capable of infecting bacteria. Virulent bacteriophages can cause bacteria to lyse and die. Bacteriophages are reported as the most diverse organisms in the biosphere. Unlike the broad-spectrum antibiotics, bacteriophage specific bactericidal function has little effect on the normal flora in the body (Rozand and Feng, 2009; Oliveira et al., 2016). Moreover, phages are capable of penetrating the inner layers of the biofilms and infecting dormant cells, considered to be a significant advantage of phages over antibiotics in killing biofilms. Phage therapy consists of the use of a single phage preparation, as well as a cocktail of multiple phage (Zhang et al., 2018). Since bacteriophage therapy exhibits high efficiency in treating bacterial infections, phages have been extensively applied as antibacterial agents in food production and aquaculture industry.

Bacteriophages lytic against other types of bacteria (e.g., *Escherichia coli* (Sadekuzzaman et al., 2017; Scanlan et al., 2017; Gilcrease and Casjens, 2018), *Pseudomonas aeruginosa* (Tsao et al., 2018; Bru et al., 2019) and *Streptococcus* (Leprohon et al., 2015; Lavelle et al., 2018; Szymczak et al., 2019) have been suggested to effectively disrupt mono-biofilms formed by their respective hosts. In this study, the morphological characterization and full genomic of a novel lytic bacteriophage, IME-JL8, were presented. According to the sequencing and analysis of the IME-JL8 genome, the lack of bacterial virulence or lysogenesis-related ORFs was revealed, so this bacteriophage was proven eligible for use in phage therapy. Besides, the phage can disrupt existing *C. freundii* biofilms. Furthermore, as suggested from the positive results of this study, phage treatment may act as an effective approach and exhibit high potential to prevent and treat *C. freundii*-related disease.

MATERIALS AND METHODS

Animal Feeding

In this study, specific pathogen-free and clinically healthy common carp (average weight 50 ± 1 g) specimens were provided by a commercial fish farm and employed for subsequent studies. Fish were maintained in 200 L flow-through tanks at 25 ± 1°C under natural photoperiod. Fish were fed with commercial diet twice a day at a feeding rate of 1% body weight.

Bacterial Strains and Phages

Table 1 lists the bacterial strains used in the present study. The antibiotic sensitivity test on *C. freundii* CF8 was showed in

TABLE 1 | Bactericidal spectrum of IME- JL8.

Bacterial strain	Date of collection (Year-Month)	Efficiency of plating of IME-JL8
Citrobacter freundii 2052[2]	_[5]	N
Citrobacter freundii 2262[2]	_[5]	N
Citrobacter freundii 19[2]	_[5]	N
Citrobacter freundii 78[2]	_[5]	N
Citrobacter freundii 2151[2]	_[5]	N
Citrobacter freundii 1152[2]	_[5]	N
Citrobacter freundii 15[2]	_[5]	N
Citrobacter freundii 1136[2]	_[5]	N
Citrobacter freundii 77[2]	_[5]	N
Citrobacter freundii 1025[2]	_[5]	N
Citrobacter freundii 223[2]	_[5]	N
Citrobacter freundii 1864[2]	_[5]	N
Citrobacter freundii CF8[1]	2016–2011	Y
Citrobacter freundii CF2[1]	2016–2011	N
Citrobacter freundii CF3[1]	2016–2011	N
Escherichia coli ATCC 25922[3]	_[5]	N
Staphylococcus aureus ATCC 25923[3]	_[5]	N
Bacillus subtilis ATCC14579[3]	_[5]	N
Salmonella sp. ATCC10248[3]	_[5]	N
Klebsiella pneumoniae BAA-2146[3]	_[5]	N
Streptococcus sp. CVCC606[4]	_[5]	N

[1]Isolated from the carp kept in our lab (Changchun, Jilin province, China). [2]Given by professer Yigang Tong. [3]Purchased from American Type Culture Collection (ATCC). [4]Purchased from China Institute of Veterinary Drug Control (CVCC). [5]No data are available. [6]Y, yes; N, No.

Supplementary Table S1. The *C. freundii* strains were incubated in 30% glycerol at −80°C and subsequently cultured in Brain-Heart Infusion (BHI) broth at 37°C.

In the present study, *C. freundii* CF8 was adopted for phage propagation. Sewage samples of 500 mL were harvested from the sewerage systems in Changchun, China. The bacteriophage was counted and purified with the double-layer agar plate method. To be specific, the phage was purified via repeated double-layer agar plate till the plaques became homogeneous. Spot tests were performed to identify the presence of phage, and the phages were amplified and incubated at 4 and −80°C in glycerol (3:1 [v/v]).

Host Range Determination

This study characterized the host range of the phage with the double-layer agar plate method following the previous description with minor modification (Yang et al., 2015). **Table 1** elucidates the bacterial strains used in the study.

Biological Characteristics of the Phage

On the whole, the biological characteristics of phage IME-JL8 consist of the killing curves of the phage under a range of multiplicities of infection (MOI), One-step growth, thermal stability, pH stability, as well as storage stability. To determine phage titer and MOI, the double-layer agar plate method was employed, and plaques were measured after the strains were cultured for 8 h. The maximum MOI refers to the optimal MOI for this bacteriophage (Al-Zubidi et al., 2019).

The one-step growth curve of the phage was determined following the previous description with some modifications (Al-Zubidi et al., 2019). In summary, the *C. freundii* CF8 strain (mid-exponential phase) was cultured, harvested and resuspended in fresh BHI broth. The phage was added at a MOI for 10 min adsorption at 37°C. The phage–host mixture underwent the centrifugation at 6797 × *g* for 10 min at 4°C. The pellets were suspended in 10 mL of fresh BHI medium that was preheated at 37°C. Then, the achieved suspension was incubated at 37°C while being shaken at 140 rpm. Samples were taken at 10 min intervals over 160 min, immediately diluted and subsequently plated for phage titration with a double-agar layer technique.

The susceptibility of phage IME-JL8 to varying pH was determined by incubating the bacteriophage in BHI broth adjusted to pH 4–10 for 1 h, complying with the previous description (Cai et al., 2019). The thermal stability of phage IME-JL8 was determined by incubating IME-JL8 in BHI broth at 4, 25, 37, 50, 60, 70, and 80°C for 80 min. Moreover, to test phage stability after long-term storage, aliquots of phage IME-JL8 suspensions were incubated at 4°C for 1 year. Furthermore, a double layer agar plate was employed in the pH stability, thermal stability and storage stability assay.

Phage Morphology and SDS-PAGE Analysis of Structural Proteins

The concentration and purification of phage were conducted following the previous description with minor modifications (Gong et al., 2016). After the IME-JL8 phage was cultured in a large scale, BHI lysates underwent the centrifugation at 6797 × *g* for 10 min at 4°C to remove cell debris. In the presence of 10% polyethylene glycol 8000 and 1 M NaCl, phage particles were precipitated from culture supernatant and subsequently dissolved in 5 mL PBS. The phage suspension was placed on the top of a discontinuous CsCl gradient (1.45, 1.50, 1.70 g/mL) and underwent centrifugation at 126,100 × *g* for 3 h at 4°C. The phage band was harvested and dialyzed. Next, a sample was applied to copper grids stained with phosphotungstic acid (PTA, 2% w/v) negatively and characterized under a transmission electron microscopy (TEM) (JEOL JEM-1200EXII, Japan Electronics and Optics Laboratory, Tokyo, Japan) at an accelerating voltage of 80 kV.

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to define the major proteins in bacteriophages IME-JL8. The concentrated phage particles were mixed with the sample buffer (supplemented by 2 mM 2-mercaptoethanol), and the samples were heated at 95°C for 7 min. Afterwards, 10 μL of lysate was loaded directly onto standard 15% SDS-PAGE gels for protein separation. Next, the gels were stained with Instant Blue (Expedeon).

Phage Genome Sequencing

With the Universal Phage Genomic DNA Extraction Kit (Knogen, Guangzhou, China), genomic phage DNA was isolated from produced high titer phage particles ($\geq 10^{10}$ PFU/mL) and incubated at −20°C for sequencing. Based on Illumina HiSeq 2500 sequencing, the whole genome sequencing of phage IME-JL8 was performed by Suzhou GENE-WIZ Biotechnology Co., Ltd. The genome sequences were assembled with the SOAP denovo package. With CGView[1], the circle map of the IME-JL8 genome was generated. Genome annotation and potential open reading frames (ORFs) were assessed and analyzed with BLAST and GeneMarkS. Next, global genome comparisons were drawn with Mauve (version 2.3.1).

Biofilm Assay

Based on a 96-well microtiter plate method presented in one existing study (Zhang et al., 2018) with minor modifications, the ability of *C. freundii* CF8 strain to form biofilms was assessed. In brief, the CF8 strain was inoculated into 5 mL of sterile TSB medium and grown for 16 h at 37°C. A 1:100 dilution of culture was transferred into fresh BHI medium, and 200 μL (10^6 CFU/mL) of the culture was introduced to the wells in untreated 96-well microtiter plates. The wells supplemented by only BHI medium acted as the negative controls. The plates were incubated for 24 h at 37°C without being shaken. The medium was renewed per 12 h and then underwent the phage treatment at a final titer of 7 log10 PFU. MIC sensitive antibiotics cefoperazone sodium, tetracycline and phosphate-buffered saline (PBS) were used as the control group under the same conditions (Akturk et al., 2019). The ability of different concentrations of cefoperazone sodium and tetracycline to remove biofilms was measured. Samples received the further incubation at for 6 h 30°C. After different group treatment, each well was washed with PBS for three times and then air-dried. After the well was washed with PBS, 98%

[1]http://wishart.biology.ualberta.ca/cgview/

methanol was added and left for 10 min. Then the methanol was removed, and the plates were air-dried again. Overall, the samples were stained with 1% crystal violet solution for 45 min followed by elution with 33% acetic acid. The OD value sample was identified by a spectrometer at a wavelength of 590 nm.

In the SEM assay, the *C. freundii* CF8 strain was cultured in 5 mL of BHI to an OD_{600} of 0.6 at 37°C while being shaken at 180 rpm. The cultures were centrifuged and resuspended in BHI medium exhibiting equal volumes. Subsequently, 200 μL of bacterial culture and 200 μL of fresh BHI were added to each well in a 24-well microtiter plate; the wells were covered with sterile 14-mm-diameter glass sheets that were preadministrated with polylysine. Next, the 24-well microtiter plate underwent the incubation without being shaken 24 h at 37°C. After incubation for 24 h, the non-adherent cells were removed, and the wells were washed three times with sterile PBS. The biofilms were administrated with 7 log10 PFU of phage IME-JL8 diluted in buffer for 6 h at 37°C. PBS acted as a negative control. The biofilm lysates were immobilized with 5% glutaraldehyde, dehydrated under a range of concentrations (i.e., 20, 50, 70, 90, and 100%) of ethanol, and then freeze-dried before the scanning electron microscopy (SEM) was used for characterization (Hitachi S-3400N; Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

Antimicrobial Activity of the Phage IME-JL8

For *in vitro* lysis assays, the *C. freundii* CF8 strain was cultured to log phase in BHI broth (OD_{600} = 0.6) and cleaned three times with PBS. Next, the produced IME-JL8 dilutions in PBS buffer were inoculated into double-strength BHI. The mixture was diluted to normal strength BHI and then normalized to a final bacterial count of 10^8 CFU/mL; meantime, phage was added at a MOI of 0.01 in the tubes. The cell viability for CFU/ml following exposure was measured at 10, 20, 30, 40, 50, and 60 min after incubation. Bacterial growth without phage addition acted as the control. The number of viable CF8 cells (CFU/mL) was ascertained by serial dilution and plated on BHI agar plates.

The therapeutic potential of phage was assessed for its ability to treat the infection of *C. freundii* in fish. First, the carps were randomly split into two groups. Carps in one group were injected intraperitoneally with 200 μL / fish of phage IME-JL8 (1×10^8 PFU/mL), while the other group was the blank control administrated with PBS over a 10-day follow-up. To determine the bacterial dose leading to 100% mortality over a 7-day follow-up (the minimal lethal dose [MLD]), groups of 6 carp per experiment were injected intraperitoneally (i.p.) with different inocula of *C. freundii* CF8 (10^7, 10^8, 10^9, 10^{10}, and 10^{11} CFU, 100 μL/fish). The number of dead fish was recorded daily. Once the MLD had been determined, 2 × MLD acted as the infective inoculum (challenge dose).

Following infection with 2 × MLD (2×10^9 CFU/ fish, 100 μL/fish) of *C. freundii*, 1 h later, the fish were treated intraperitoneally with IME-JL8 at different concentrations (1×10^6, 1×10^7 or 1×10^8 PFU/mL, 100 μL/fish) (n = 6 in respective group) (Gu et al., 2016; Cheng et al., 2017; Zhang et al., 2018). To further test the bactericidal ability of bacteriophages *in vivo*, the fish were infected with 2 × MLD (2×10^9 CFU/ fish, 100 μL/fish) of *C. freundii*, 12 and 24 h later, the fish were treated intraperitoneally with IME-JL8 at concentrations (1×10^8 PFU/mL, 100 μL/fish) (n = 6 in respective group). The control group was administrated with an equal amount of PBS (100 μL/fish) buffer under the identical conditions. The survival rate was recorded per day for 7 days.

The concentrations of the cytokines (TNF-α, IFN-γ, and IL-1β) in the blood samples in different groups at 1, 6, 12, or 24 h after being challenged were quantified with qPCR based on the methods of existing studies. All qPCRs samples were performed with three replicates. The primers for the immune-related genes studied and β-actin, as examined as a housekeeping gene (**Table 2**).

Data Analysis

SPSS version 13.0 (SPSS, Inc., Chicago, IL, United States) was employed to statistically analyze other experimental data with a one-way analysis of variance (ANOVA). GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, United States). A *p-value* < 0.05 was considered to exhibit statistical significance. Error bars represent standard deviation of the mean.

RESULTS

Phage Purification and Characteristics

Based on plaque purification, *C. freundii* phage IME-JL8 was isolated from the Chang Chun sewage. As incubated with *C. freundii* CF8, IME-JL8 formed clear plaques (2–3 mm diameter) on lawns of CF8 (**Figure 1A**). Apart from CF8, IME-JL8 failed to lyse other *C. freundii* strains and other species (**Table 1**). The maximum titers of IME-JL8 could reach 10^9 PFU/mL under the MOI of 0.01 (**Figure 2C**). Thus, the one-step growth curve of IME-JL8 propagated on the CF8 strain in BHI broth was plotted at a MOI of 0.01. **Figure 2B** suggests that the mentioned one-step growth curve revealed that the latent and rise periods were approximately 30 and 40 min.

TABLE 2 | Sequences and conditions of the primers used in RT-PCR analysis.

Gene	Sequence(5′-3′)	Accession	PCR product (bp)	Cycling conditions	No. of cycles
IL-1β	F:AACTGATGACCCGAATGGAAC R:CACCTTCTCCCAGTCGTCAAA	AB010701	133	95°C–30 s 61°C–30 s	40
IFN-γ	F: AACAGTCGGGTGTCGCAAG R:TCAGCAAACATACTCCCCAG	AB376666	141	95°C–30 s 62°C–30 s	40
TNF-α	F: TTATGTCGGTGCGGCCTTC R: AGGTCTTTCCGTTGTCGCTTT	AJ311800.2	101	95°C–30 s 63°C–30 s	40
β-actin	F: CAAGATGATGGTGTGCCAAGTG R:TCTGTCTCCGGCACGAAGTA	M24113.1	352	95°C–30 s 62°C–30 s	40

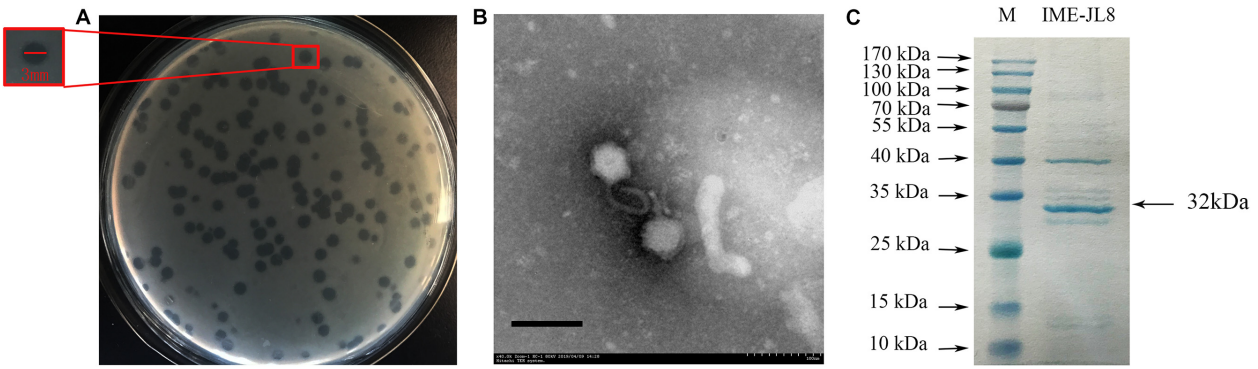

FIGURE 1 | Phage morphology and structural protein detection. **(A)** Bacteriophage IME-JL8 spotted onto *C. freundii* CF8 culture on BHI agar. Each single plaque was ≈3 mm diameter. **(B)** TEM image of IME-JL8 revealing *Siphoviridae* bacteriophage with long tail and icosahedral head. **(C)** SDS-polyacrylamide gel (15%) electrophoresis of IME-JL8 structural proteins. M: molecular mass marker. Lane 1, IME-JL8 proteins. The most abundant structural protein was about 32 kDa.

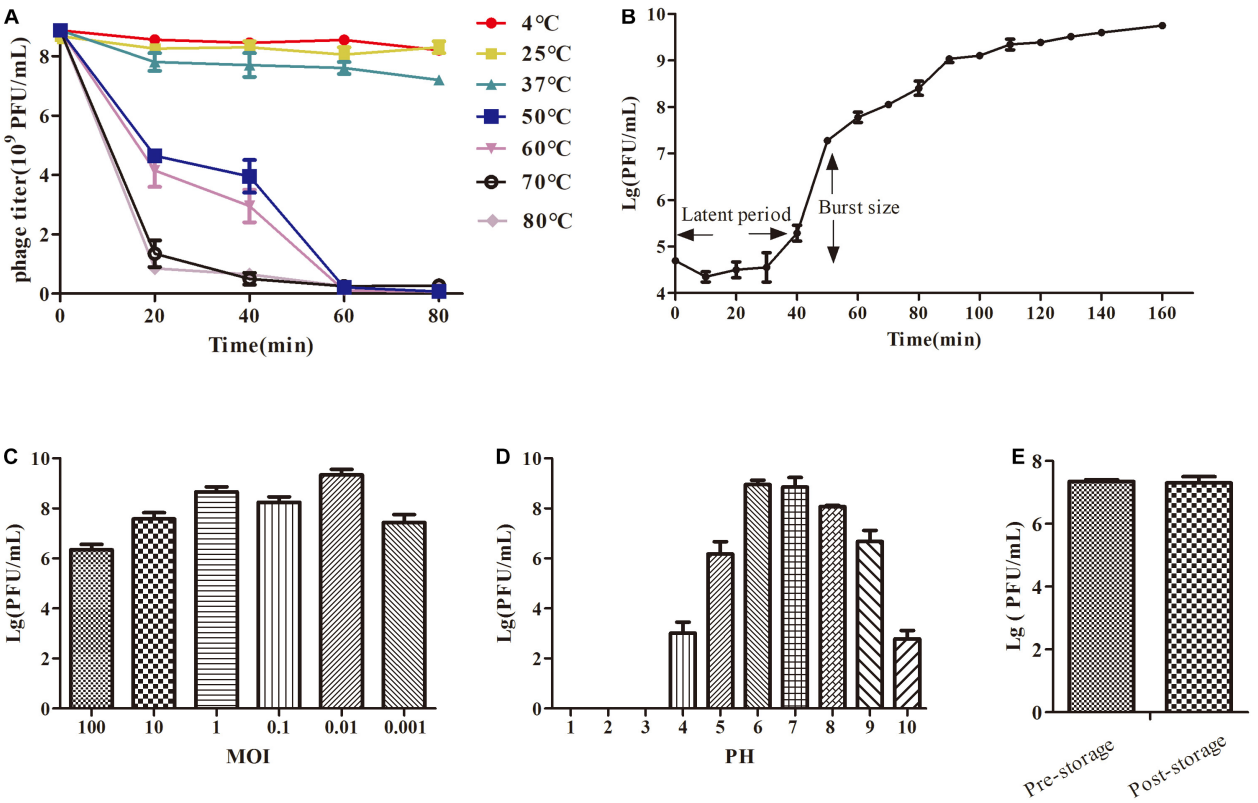

FIGURE 2 | The growth characteristics and stability tests of IME-JL8. **(A)** Thermal stability: phage particles were incubated at different temperatures as indicated. **(B)** A one-step growth curve of IME-JL8. **(C)** Titers of the phage under different MOI (phage/bacteria = 0.001, 0.01, 0.1, 1, 10, and 100), as represented in the *Y*-axis. At the MOI of 0. 01, IME-JL8 peaked the maximum titers. **(D)** pH stability: IME-JL8 were incubated under different pH values. **(E)** Long-term storage stability: the titer of pre-storage and post-storage (for 1 year) of IME-JL8.

Phage IME-JL8 can basically maintain its original activity between 4, 25, and 37 °C (**Figure 2A**). Simultaneously, IME-JL8 can maintain high activity at pH 4–10 (**Figure 2D**). Under pH below 4 or above 10, the phage activity declined sharply. Furthermore, the titer of IME-JL8 almost remained unchanged after 1 year of storage at 4°C (**Figure 2E**).

From the structural perspective, the transmission electron microscopy revealed that IME-JL8 exhibited morphological characteristics of the family *Siphoviridae*, with a long flexible tail and an icosahedral head. The diameter of the isometric head nearly reached 68 nm (**Figure 1B**). To characterize the IME-JL8 phage in depth, the structural protein composition

was analyzed by SDS-PAGE. Following gel electrophoresis, 11 major protein bands were visualized by Coomassie staining (**Figure 1C**). The size of the most abundant major structural protein reached about 32 kDa.

Overview of the Phage IME-JL8 Genome

To elucidate this phage at the genetic level, the complete genome sequence of IME-JL8 was sequenced, analyzed and then deposited in GenBank under accession number (MT 023084). The IME-JL8 phage genome was a 49,838 bp contiguous sequence of double-stranded DNA, linear, and the overall G + C content was 47.96%. The complete genome of IME-JL8 encoded 80 assessed open reading frames (ORFs), and the arrangement of the mentioned putative ORFs was mapped at the whole-genome level (**Figure 3A**). The gene-coding potential of the global genome was 92.64%, with an average ORF size of 539.45 bp. Among these ORFs, 32 (40 %) of the initiation codons were ATG, 27 (33.75%) of the initiation codons were TTG, 13 (16.25 %) starting codons were GTG, and 8 (10 %) was GCG (**Figure 3A**).

All assessed proteins were examined for similarity to known bacterial and phage sequences deposited in the public National Center for Biotechnology Information (NCBI) databases. The modular organization of the IME-JL8 genome displayed four major conserved patterns, i.e., gene expression, gene synthesis, host lysis and virion assembly. The maximum ORF of IME-JL8 was ORF 9 (33.12899 kDa), adjacent to the structure proteins and exhibiting 99% identity with phage SRT8 (putative ATP-dependent helicase). Moreover, there were two other ORFs (i.e., ORF68 and ORF69), encoding a putative endolysin and holin protein. However, no ORFs were associated with drug resistance or lysogeny (e.g., site-specific integrases and repressors). As revealed from bioinformatics annotation and analysis, no similarities were identified between genes or proteins encoded by IME-JL8 and genes or proteins for other factors known to impact virulence during acute or chronic infection by *C. freundii*.

The full-length genome of IME-JL8 was blasted and then analyzed with the genes in the GenBank database. As revealed from a comparative analysis, the genome was highly similar to that of the *Escherichia* phage SRT8, vB_EcoS_SH2, JMPW1, JMPW2, *Shigella* phage pSf-2, *Citrobacter* phage CF1, *Salmonella* phage phSE-5, phSE-2 (**Figure 3B**). According to nucleotide blast, the genome does not exhibit 100% identical sequence homologies to currently known phage strains in GenBank, suggesting that it is a novel phage strain.

The Bactericidal Effect of the Phage IME-JL8 *in vitro*

To experimentally determine the bactericidal activity of IME-JL8 *in vitro*, the time killing assay was performed. As demonstrated from the results of the lytic assay, when IME-JL8 were incubated with CF8, the colony count decreased by 6.8 log units at 20 min after treatment (**Figure 4**).

The ability of CF8 to form biofilms was experimentally determined. We found that CF8 formed the most stable biofilms at 24 h in the previous result (OD_{590} = 0.374, 6 h; OD_{590} = 0.628, 12 h; OD_{590} = 0.927,24 h; OD_{590} = 0.784, 48 h; OD_{590} = 0.703, 72 h). **Figure 5A** illustrates the reduction of biofilm after phage treatment with titers of 7 log10 PFU/mL for 6 h. The CF8 biofilm removal activity of 77.7% observed when phage IME-JL8 was applied to a final titre of 7 log10. Two sensitive antibiotics cefoperazone sodium and tetracycline were selected depending on their mechanism of action: cell wall synthesis inhibitor (cefoperazone sodium), protein synthesis inhibitor (tetracycline). When we used MIC cefoperazone sodium and tetracycline as the control group to remove biofilms, the CF8 biofilms removal activities were 30.7 and 20.3%, respectively (**Figure 5A**). When using 32 × MIC cefoperazone sodium and tetracycline, the CF8 biofilms removal activities were 82.5% (**Supplementary Figure S1**). Furthermore, micrographs captured under the scanning electron microscopy (SEM) clearly indicated the structurally complex variation, and the number of live cells in the PBS treatment group was found significantly higher than in the phage IME-JL8 treatment group (**Figures 5B,C**).

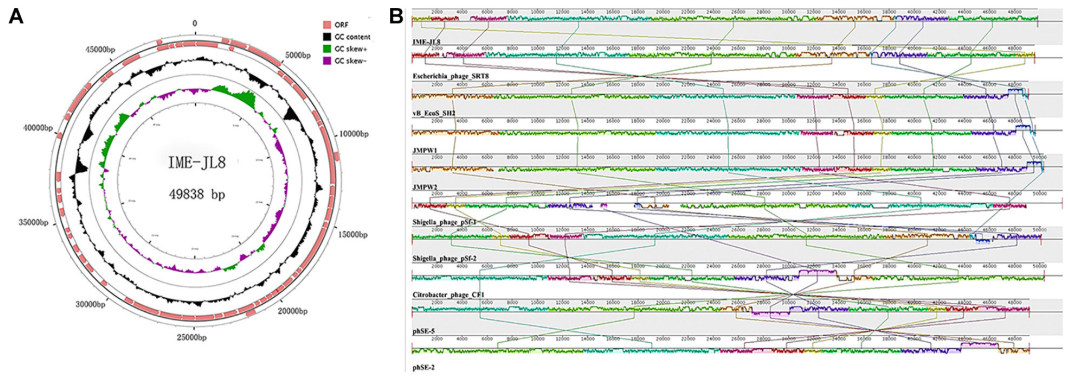

FIGURE 3 | Genome characteristics of IME-JL8. **(A)** The genetic and physical organization of the IME-JL8 genome. ORFs with 80 or more residues are represented by arrows and the arrowheads point in the transcription direction. The figure also maps the G+C content, which is skewed. **(B)** Multiple genome alignments among IME-JL8 and other homologous phages. Similarity is indicated by the height of the bars, complying with the average level of conservation in that region of the genome sequence. The homologous phages are marked in the picture.

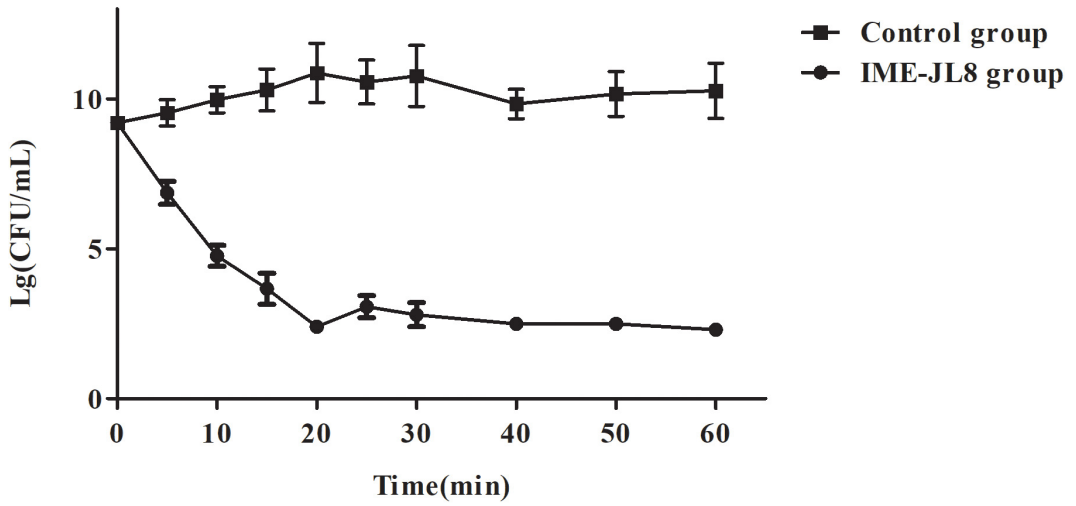

FIGURE 4 | The bactericidal activity of IME-JL8 to *C. freundii* CF8. The CFU/mL decrease of the *C. freundii* CF8 culture was adopted to assess the bactericidal activity of IME-JL8. A final bacterial (*C. freundii* CF8) count of 10^8 CFU/mL and phage IME-JL8 was added at a MOI of 0.01 in the tubes. The bacterial administrated with PBS as control. Each data is expressed as mean ± SD from three biological experiments.

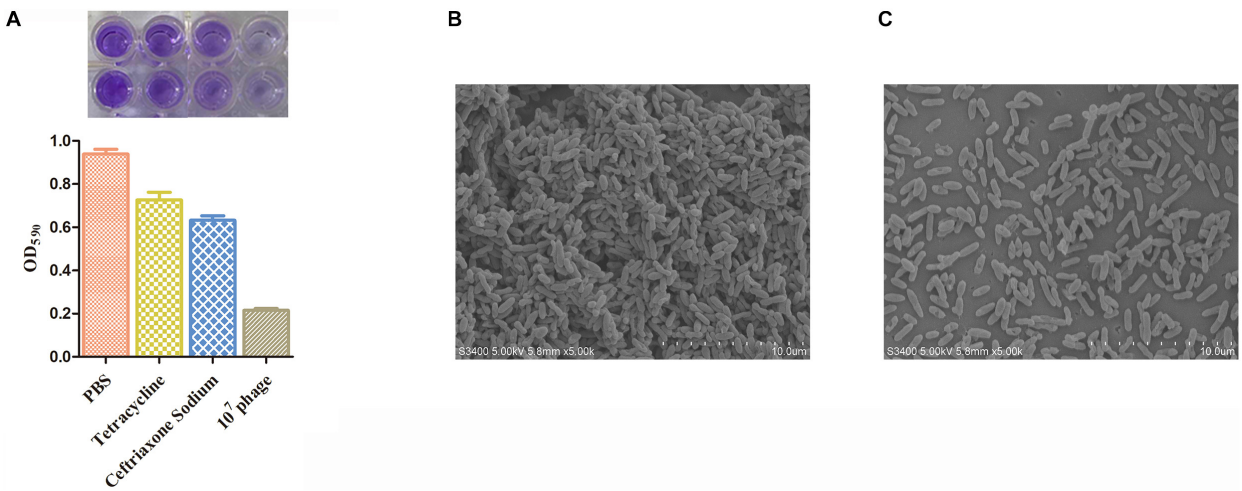

FIGURE 5 | Biofilm removal activity of IME-JL8. The reduction of biofilm after phage treatment with titers of 7 log10 PFU/mL and MIC concentration of cefoperazone sodium and tetracycline for 6 h. **(A)** Biofilm formation state was indicated by OD_{590} values. **(B,C)** Micrographs of biofilms taken under the scanning electron microscopy. **(B)** The SEM of bacteria incubated overnight at 37°C. **(C)** The SEM of bacteria administrated with IME-JL8 for 6 h. The bars represent 10 μm.

Phage Therapeutic Study

In the safety test, the fish exhibited the survival rate of 100%, demonstrating that IME-JL8 had no side effects on carps. Intraperitoneal injection of 2 × minimum lethal dose (MLD) (2 × 10^9 CFU/carp) of *C. freundii* CF8 was sufficient to produce a 100% mortality rate within 3 days, in contrast, all the carps administrated with phage IME-JL8 (1 × 10^8 PFU/mL, 100 μL/carp) recovered (**Figure 6A**). After infection CF8 (2 × 10^9 CFU/carp) for 1 h, the bacterial loads reached $>10^6$ CFU/mL in the blood (**Figure 6B**). At this time, the carp were administrated with phage IME-JL8 (1 × 10^8 PFU/mL, 100 μL/carp). As shown in **Figure 6B**, the bacteremia greatly decreased reaching approximately 3.3 log units after 12 h in the blood. By contrast, the bacterial loads in the carps administrated with PBS reached approximately 8.6 log units after 12 h in the blood. After infection CF8 (2 × 10^9 CFU/carp) for 12 h, the bacterial loads reached $>10^8$ CFU/mL in the blood (**Figure 6D**). At this time, the carp were administrated with phage IME-JL8 (1 × 10^8 PFU/mL, 100 μL/carp). As shown in **Figure 6C**, phage IME-JL8 was sufficient to produce a 45% survival rate within 3 days. The bacteremia greatly decreased reaching approximately 3.7 log units after 24 h in the blood. By contrast, the bacterial loads in the carps administrated with PBS reached approximately 9.7 log units after 24 h in the blood (**Figure 6D**). However, for the 24 h post-treatment, the fish died within 2 days in the phage IME-JL8 treated group (**Figure 6E**)

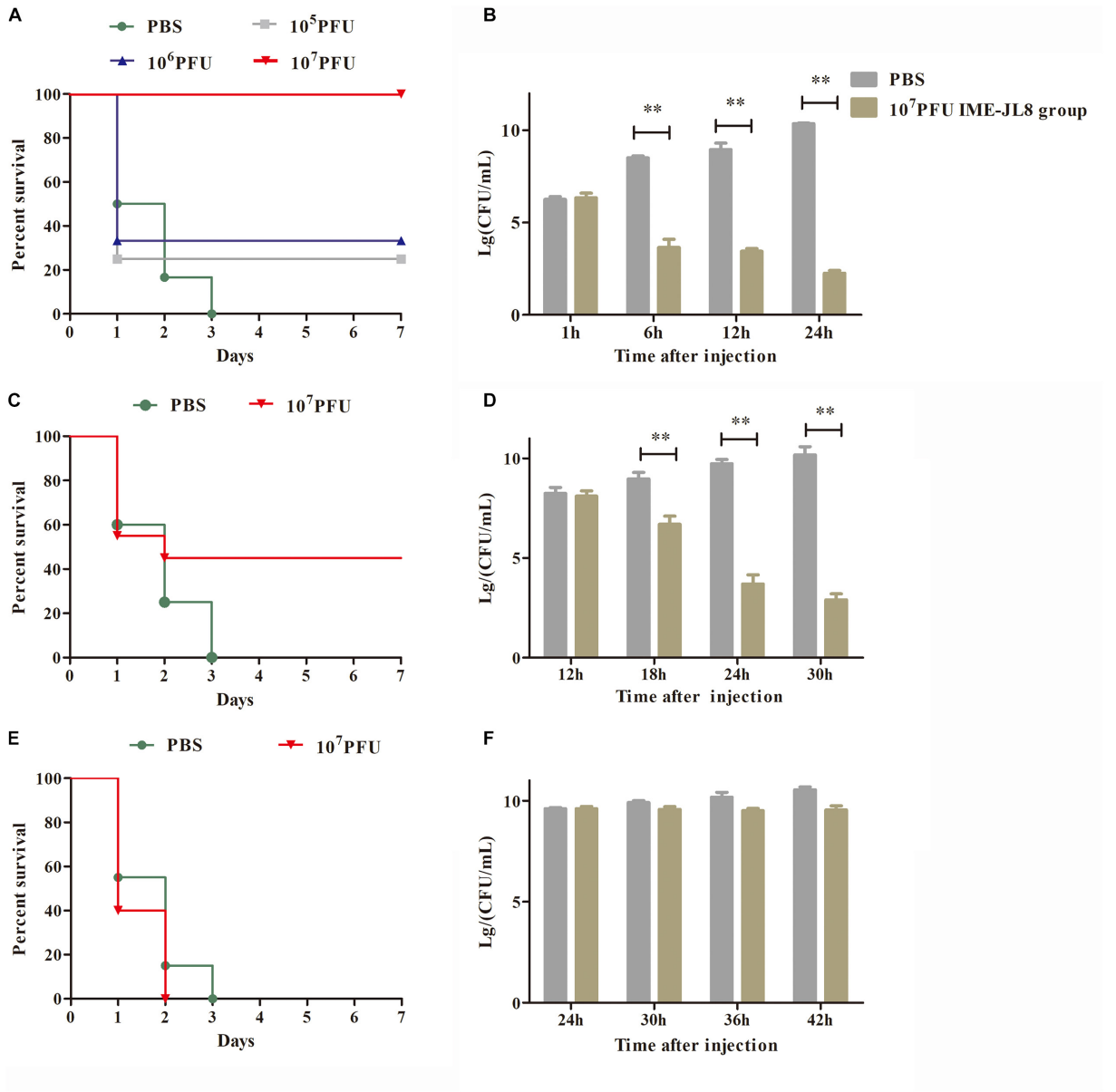

FIGURE 6 | IME-JL8 therapeutic study. The fish were intraperitoneal injection of 2 × minimum lethal dose (MLD) (2 × 10^9 CFU/carp) of *C. freundii* CF8. **(A–E)** Survival rate of different groups. One hours later, 10^5, 10^6, and 10^7 PFU of IME-JL8 were introduced intraperitoneal **(A)**. After injection of *C. freundii* CF8 (2 × 10^9 CFU/carp) 12 h later **(C)**, 24 h later **(E)**, 10^7 PFU phage of IME-JL8 were introduced intraperitoneal. Control fish were administrated with PBS under the identical conditions. **(B,D,F)** Colony counts of bacteria changed in the blood. After injection of *C. freundii* CF8 (2 × 10^9 CFU/carp) 1 h later **(B)**, 12 h later **(D)**, 24 h later **(F)**. colony counts of bacteria changed in the blood at regular intervals (n = 6 in each group). Control fish were administrated with PBS under the identical conditions. The means and standard deviations are represented as points with error bars.

and there was no significant decrease in the bacterial load in the blood (**Figure 6F**).

By qRT-PCR analysis, the levels of TNF-α, IL-1β and IFN-γ in the blood of the different treatment groups were examined. As shown in **Figure 7**, the cytokines levels of IL-1β and IFN-γ in the blood at 6 h increased rapidly and then significantly decreased after IME-JL8 treatment. However, the cytokines levels of TNF-α peaked at 12 h and then decreased in the IME-JL8 treatment group. TNF-α, IL-1β, and IFN-γ expressions in the blood exhibited significantly lower levels at 18 h compared with the control groups.

DISCUSSION

Multidrug-resistant bacteria are becoming increasingly prevalent worldwide. *Citrobacter freundii* refers to a fish pathogen known for its ability to cause injury and high mortality

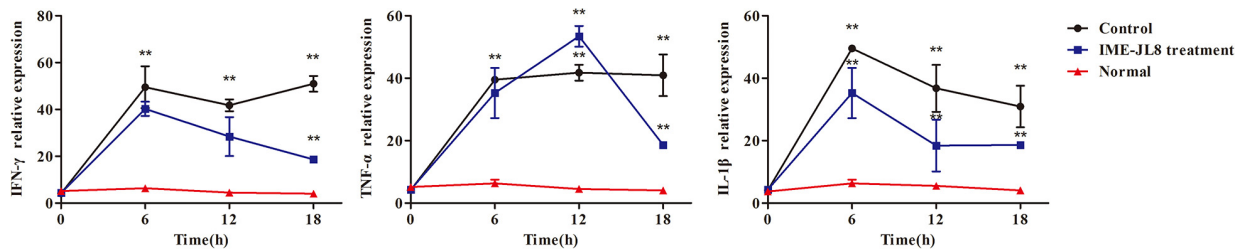

FIGURE 7 | Cytokine levels. The fish were injected intraperitoneally with *C. freundii* CF8 (2×10^9 CFU/carp). One hour later, IME-JL8 (10^8 PFU/mL, 100 μL/fish) or PBS (100 μL/fish) were introduced intraperitoneally to the fish. At the indicated times, the levels of TNF-α, IL-1β and IFN-γ in the blood of the different treatment groups were determined by qRT-PCR. ***P* values of <0.05 compared with the PBS treated control. The mentioned experiment was repeated three times. Each data is expressed as mean ± SD from three biological experiments.

(Yang et al., 2018). For the study, the clinical isolate *C. freundii* CF8 applied in the present study demonstrated a high resistance to antibiotics and was a robust biofilm producer. It is known that multi-resistant and strongly biofilm forming strains have virulence factors, probably contributing to the strain pathogenicity and to the difficulty in treating the disease in fish (Zurfluh et al., 2017). Accordingly, precaution measures are required to manage infections by *C. freundii* strain.

In the present study, our results showed that a newly isolated bacteriophage IME-JL8 can lyse the actively growing cells of *C. freundii* efficiently. Phages can be isolated from a wide variety of sources e.g., sea water, sewage water/sludge ponds etc. (Abedon, 2015; Xu et al., 2015; Jurac et al., 2019). Phage IME-JL8 was isolated from sewage. Sewage is a rich source of phages that infect pathogenic bacteria, e.g., *E. coli, P. aeruginosa*, and *Salmonella*. Thus, it acts as an appropriate sample source for conventional bacterial phage isolation. Physical and chemical stability are critical for phages to be applied for clinical antimicrobial preparations. In the present study, the titers of IME-JL8 exhibited stability in the floating range of conventional pH (5–9) and temperature (<50°C), and the titer of this phage could be long maintained at 4°C. Electron microscopy indicated that IME-JL8 had morphological characteristics of the family *Siphoviridae*, with a long flexible tail and an icosahedral head. This is different from the *C. freundii* phage that has been discovered (Chaudhry et al., 2014; Zhao et al., 2016). The *C. freundii* phage they found were all short tails. Similar to these two phages, IME-JL8 was also highly specific to the bacteria, only sensitive to their host bacteria. Moreover, antibiotic resistance genes or putative virulence factors were not reported in this phage by genomics analysis (Debarbieux et al., 2018; Santos et al., 2018; Straus and Bo, 2018). All the mentioned factors strongly suggest that IME-JL8 could be a potential therapeutic phage against multiple *C. freundii* infections.

Besides biological characteristics, the present study also analyzed the genomic information of IME-JL8. The genome size of IME-JL8 is 49,838 bp, which is less than that of other *C. freundii* phage no. KM236237 (178,171 bp) and no. KT001915 (172,733 bp) (Hwang et al., 2015; LeSage et al., 2015). Similar to these two phages, we did not find putative virulence factors in IME-JL8. Comparative genome analyses revealed the close associations of *Escherichia* phage SRT8, vB_EcoS_SH2, JMPW1, JMPW2, *Shigella* phage pSf-2, *Citrobacter* phage CF1, *Salmonella* phage phSE-5, phSE-2 and suggested molecular clues to elucidate host adaptations in the relevant phages mentioned.

Since bacterial biofilms are highly resistant and resilient to conventional antibacterial therapy, it has been difficult to combat the mentioned infections. An innovative alternative to the bio-control of bacterial biofilms could be the use of bacteriophages, which are specific, non-toxic and self-proliferating, as well as capable of penetrating into biofilms (Kabwe et al., 2019). In this report, the capacity for IME-JL8 to disrupt established *Citrobacter freundii* biofilms was demonstrated. As suggested from the mentioned results, phage therapy on 24-well microplate can effectively reduce the biofilms. It has been reported that treatment with the phage eradicated post-treated biofilm in (44–63%). Crystal violet (CV) was adopted to stain the biofilms; subsequently, the OD_{590} was determined. Phages for *Actinomyces naeslundii*, *Enterococcus faecalis*, *Fusobacterium nucleatum*, *Lactobacillus* spp., *Neisseria* spp., *Streptococcus* spp., and *Veillonella* spp. were isolated and characterized; then, they were reported to effectively reduce the biofilms (Abedon, 2016; Hansen et al., 2019; Issa et al., 2019). In this part, we used two sensitive antibiotics cefoperazone sodium and tetracycline as control group to analyze the effect of antibiotics on bacteria in the biofilm state. The results showed that when we used MIC cefoperazone sodium and tetracycline to remove biofilms, the CF8 biofilms removal activities were 30.7 and 20.3%, respectively (**Figure 5A**). The phenomenon was observed between the concentrations of cefoperazone sodium and tetracycline on the biofilm killing eradication. The biofilm killing eradication rate increased with the increase of antibiotic concentration. When using 32 × MIC cefoperazone sodium and tetracycline, the CF8 biofilms removal activities was the same as phage-treated group (**Supplementary Figure S1**). The results were similar to those of previous studies (Akturk et al., 2019). It was necessary to increase gentamicin concentration to obtain a similar killing effect as occurs in the bacteria with biofilms.

To assess the therapeutic effect of IME-JL8, different phage doses were administered intraperitoneally 1 h after the injection. All the carps administrated with phage IME-JL8 (1×10^8 PFU/mL, 100 μL/carp) recovered (**Figure 6A**). After infection CF8 (2×10^9 CFU/carp) for 1 h, the bacterial loads reached $>10^6$ CFU/mL in the blood (**Figure 6B**). Meantime,

the carp were administrated with phage IME-JL8 (1×10^8 PFU/mL, 100 μL/carp). **Figure 6B** suggested that the bacteremia significantly decreased, reaching approximately 3.3 log units after 12 h in the blood. However, after infection CF8 (2×10^9 CFU/carp) for 12 and 24 h, phage IME-JL8 was sufficient to produce a 45% survival rate within 3 days for 12 h. For the 24 h post-treatment, the fish died within 2 days in the phage IME-JL8 treated group and there was no significant decrease in the bacterial load in the blood. The results showed that phage treatment was effective within 12 h of bacterial infection of fish. The levels of TNF-α, IL-1β, and IFN-γ in the blood of the different treatment groups were determined experimentally by qRT-PCR analysis. The levels of three cytokines increased and peaked at 12 and 6 h, respectively, then decreased. TNF-α, IL-1β, and IFN-γ expressions in the blood exhibited significantly lower levels at 18 h compared with the control groups. The phenomenon is similar to the cytokine secretion of phage X1 (Xue et al., 2020) and phage VB-SavM-JYL01 (Ji et al., 2019) in the treatment of *Yersinia* and *S. aureus*. Some studies have shown that phages have anti-inflammatory properties that reduce the secretion of pro-inflammatory factors. When serum of mice infected with *S. aureus* was treated with phage, the production of TNF-α and IL-6 were reduced, and this phenomenon was also observed in serum samples from some patients treated with phage therapy for bacterial infection (Leung and Weitz, 2017). However, the detailed mechanism of how bacteriophages induce anti-inflammatory effects remains unclear.

During phage therapy, safety is primarily concerned with for therapeutic application on farms or in the food chain. Serious side effects from endotoxin have been rarely reported in the early literature concerning experimental phage therapy. Furthermore, in the present study, the safety of IME-JL8 was determined experimentally before the treatment and prevention trial. The survival rate of the fish reached 100%, demonstrating that lysates of phage are safe and reliable.

Since bacteriophage exhibits rigorous host specificity, and bacteria will develop resistance to bacteriophage in mutual evolution with bacteriophage, single bacteriophage preparation cannot satisfy the treatment requirements. The phage cocktail preparation (cocktail therapy) and personalized phage treatment method may be one of the subsequent research directions of phage treatment (Heyse et al., 2015; Kim et al., 2017; Cieplak et al., 2018). Moreover, some bacteriophages carry resistance genes and even virulence genes, making them unlikely to use directly for treatment. Thus, the isolation, identification, whole genome sequencing and genomic analysis of the bacteriophage are of high significance. It can obtain comprehensive and clear genetic information and evolution information of the bacteriophage, as well as theoretically underpinning bacteriophage therapy.

CONCLUSION

In summary we isolate a newly phage IME-JL8 using a *C. freundii* strain originating from the fish as the host. We have identified its biological characteristics as well. The efficient and lytic activity and genomic characteristics of IME-JL8 make it eligible for use in phage therapy. Indeed, IME-JL8 exhibits great therapeutic potential for the treatment in fish caused by *C. freundii*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

LZ: conceptualization. NY and XZ: formal analysis. RC and JT: investigation. YZ and YK: project administration. AQ: resources. YL, WS, JS, XS, and JY: software. GW and LZ: Supervision. KJ: writing – original draft. SR: writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.585261/full#supplementary-material

Supplementary Figure 1 | Biofilm removal activity of antibiotics. The reduction of biofilm after two antibiotics treatment with different concentrations (MIC, 2 × MIC, 4 × MIC, 8 × MIC, 16 × MIC, 32 × MIC) for 6 h. Biofilm formation state was indicated by OD_{590} values. **(A)** Different concentrations of tetracycline to remove biofilm. **(B)** Different concentrations of cefoperazone sodium to remove biofilm. $^{**}P$ values of <0.05 compared with the PBS treated control. The mentioned experiment was repeated three times. Each data is expressed as mean ± SD from three biological experiments.

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frontiers in Microbiology

MINI REVIEW
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The Rationale for Using Bacteriophage to Treat and Prevent Periprosthetic Joint Infections

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Prosthetic joint infection (PJI) is a devastating complication after a joint replacement. PJI and its treatment have a high monetary cost, morbidity, and mortality. The lack of success treating PJI with conventional antibiotics alone is related to the presence of bacterial biofilm on medical implants. Consequently, surgical removal of the implant and prolonged intravenous antibiotics to eradicate the infection are necessary prior to re-implanting a new prosthetic joint. Growing clinical data shows that bacterial predators, called bacteriophages (phages), could be an alternative treatment strategy or prophylactic approach for PJI. Phages could further be exploited to degrade biofilms, making bacteria more susceptible to antibiotics and enabling potential combinatorial therapies. Emerging research suggests that phages may also directly interact with the innate immune response. Phage therapy may play an important, and currently understudied, role in the clearance of PJI, and has the potential to treat thousands of patients who would either have to undergo revision surgery to attempt to clear an infections, take antibiotics for a prolonged period to try and suppress the re-emerging infection, or potentially risk losing a limb.

Keywords: periprosthetic joint infection, phage (bacteriophage), treatment, biofilm, immune system

INTRODUCTION

Joint replacement is a life-enhancing procedure for millions of people around the world. Successful joint replacement improves quality of life by relieving pain as well as restoring function and independence (Giori et al., 2018). It is projected that by 2030 there will be approximately 500,000 hip and 3.5 million knee replacements performed annually in the United States alone (Kurtz et al., 2007). The vast majority of patients undergoing joint replacements experience near pain-free function, but an unfortunate minority experience pain and ultimately require additional surgery (Mortazavi et al., 2011). The etiologies of joint replacement failure include aseptic failures from loosening at the bone-cement, cement-implant, or bone-implant interfaces, fracture of the bone or implant, wear debris from the articulation, or poor implant position resulting in joint instability (Mulhall et al., 2006). However, septic failure (i.e., periprosthetic joint infection, PJI) is the most feared and often times the most common reason for joint replacement failure (Tande and Patel, 2014).

Periprosthetic joint infection is the leading cause of failure for knee replacements and the third leading cause for failure in hip replacements, accounting for between 15 and 25% of all revision surgeries (Kamath et al., 2015). Nearly 11,000 patients are affected by PJI yearly in the United States alone, costing over $1.6 billion in 2020 (Kurtz et al., 2012; Kamath et al., 2015). PJI can be categorized in three groups based on the timing of onset. Early PJI is classified when the infection occurs within 3 months after surgery, where delayed PJI occurs between 3 and 24 months after surgery. Late PJI is categorized when the PJI develops 24 months after the surgery occurred. Common signs and symptoms include swelling, redness, and pain localized to the joint, incisional erythema and/or drainage, as well as fever (Berbari et al., 1998; Bongartz et al., 2008; Ravi et al., 2012; Taylor et al., 2012).

During PJI, bacteria bound to an implant survive the administration of antibiotics by forming an antibiotic-tolerant biofilm, an extracellular polymeric substance of DNA, proteins, and polysaccharides (Fauvart et al., 2011; Urish et al., 2016). The subsequent treatment of PJI requires the removal of these biofilm contaminated implants (i.e., one- or two-stage revision surgery) in addition to the administration of antibiotics. The cost associated with each of these revisions is more than $25,000 and is associated with a significant morbidity as well as a one year mortality greater than 10% (Zmistowski et al., 2013; Kamath et al., 2015). Despite being the focus of research efforts for many years, treatment failure of PJI can be high with failure rates up between 20 and 50% when the implant is retained (Peel et al., 2011; Namba et al., 2013; Pourzal et al., 2016; Song et al., 2018).

Although PJI can occur in any patient, certain risk factors increase the risk of PJI. Obesity (body mass index, BMI $>$ 35 kg/m^2) was generally brought forth as a risk factor but in recent years this has been brought into question (Giori et al., 2018). Additional known factors are rheumatoid arthritis, immunosuppression, and malignancy (Berbari et al., 1998; Bongartz et al., 2008; Jämsen et al., 2009; Peel et al., 2011; Ravi et al., 2012; Taylor et al., 2012; Pourzal et al., 2016). Several studies associate PJI with poor glucose control at surgery, whereby diabetes mellitus is used as a surrogate (Malinzak et al., 2009; Cazanave et al., 2013; Namba et al., 2013; Pourzal et al., 2016). Besides disease-associated risk factors, peri-operative risk factors play an important role as well. One study has shown that hinged-knee prostheses are more frequently infected than standard replacements (Poss et al., 1984; Amanatullah et al., 2015). Additionally, postoperative complications associated with an increased risk of PJI include hematoma, superficial surgical site infection, wound drainage, and wound dehiscence (Berbari et al., 1998; Pulido et al., 2008; Aslam et al., 2010; Peel et al., 2011). Wound closure is critical, as open wounds or poorly apposed skin will more rapidly lead to bacterial colonization and subsequent infection.

Despite the existing treatment strategies for PJI such as surgical debridement and use of local and systemic antibiotics or the use of antimicrobial coatings and texturing, the presence of biofilm and the rise of antibiotic resistance limits the effectiveness of current treatment modalities. The use of bacteriophages (a.k.a., phages), viruses that specifically target bacteria, represents an alternative to therapeutic and preventative models. Understanding phage therapy begins with understanding the bacterial pathogens involved in PJI and how phage therapy can augment current treatment or prophylactic protocols.

BACTERIAL PATHOGENESIS OF PJI

Southwood et al. (1985) showed that most PJIs occurring within the first year are initiated by microorganisms introduced at the time of surgery (Popa and Dagan, 2011). This is often correlated with longer operation times (Peersman et al., 2006). Bacterial contamination occurs through either direct contact or aerosolized contamination of the prosthesis or periprosthetic tissues. Subsequently microorganisms begin colonizing the surface of the implant.

Staphylococcus is the predominant bacteria associated with PJIs and it likely seeds the joint as the implant crosses the skin (**Table 1**; Barberán, 2006; Laffer et al., 2006; Montanaro et al., 2011). Gram-positive bacteria, including *Staphylococcus aureus* and coagulase-negative *Staphylococcus* (CNS) infect between 50 and 60% of the implants (Tsukayama et al., 1996; Murdoch et al., 2001; Sendi et al., 2011). Other pathogens play an important role in PJI, including *Streptococcus* species, *Enterococcus*, and aerobic Gram-negative bacilli (**Table 1**; Chodos and Johnson, 2009; Lee et al., 2010; Rodríguez et al., 2010). Only a few contaminating microorganisms are needed to establish an infection and even fewer to establish a PJI. In a rabbit model, 10^4 colony forming units (CFU) of *S. aureus* will create an infection, but when an implant is present less than 10^2 CFU will create a PJI (Southwood et al., 1985). CNS are ubiquitous members of the human microbiome found on the skin with the most frequently identified member being *S. epidermidis* (Tripathi et al., 2020). Although less common, *Enterococcal* species account for 12–15% of early-onset PJI often as part of a polymicrobial infection (Bengtson and Knutson, 1991; Berbari et al., 1998; Cobo et al., 2011; Peel et al., 2012b; Tande and Patel, 2014).

At times a causative bacterial pathogen cannot be isolated during PJI. The inability to grow a pathogen in laboratory culture can be attributed to prior antimicrobial treatment, inadequate use of available microbiological methods, or an inability to detect and recognized the pathogen using currently available diagnostic methods (Tande and Patel, 2014). Clearly, identifying a bacterial pathogen is critical when employing phage therapy to treat or prevent PJI. In some cases, isolation of a bacterial pathogen from the intraoperative swab may enable to prepare an active individualized phage formulation.

Another mechanism of establishing a PJI is the contiguous spread of infection from an adjacent site, called hematogenous seeding (Tande and Patel, 2014). Several studies showed that peri-operative infections at a distant site, including urinary and respiratory tract, are associated with an increased risk of PJI (Berbari et al., 1998; Peersman et al., 2001; Pulido et al., 2008). This may be the result of transient bacteremia from the distant infection site during a high-risk time period. Ultimately, however, PJI originating from remote sites of infection are rare (Popa and Dagan, 2011).

TABLE 1 | Overview of the most common bacterial pathogens isolated form prosthetic joint infections and their available phages for therapeutic purposes.

Infectious bacteria	Occurrence	Number of phages available (according to NCBI)	Reference of bacterial infections
Staphylococcus aureus	++++	145	(Berbari et al., 1998, Berbari et al., 2010; Marculescu et al., 2006; Schäfer et al., 2008; Biring et al., 2009; Lee et al., 2010; Shukla et al., 2010; Kim et al., 2011; Kusuma et al., 2011; Mahmud et al., 2012; Peel et al., 2012a)
Coagulase negative *Staphylococcus*	++++		(Berbari et al., 1998, Berbari et al., 2010; Marculescu et al., 2006; Schäfer et al., 2008; Biring et al., 2009; Lee et al., 2010; Shukla et al., 2010; Kim et al., 2011)
Streptococcus species	+++	55	(Berbari et al., 1998, Berbari et al., 2010; Marculescu et al., 2006; Schäfer et al., 2008; Biring et al., 2009; Lee et al., 2010; Shukla et al., 2010; Kim et al., 2011; Kusuma et al., 2011; Mahmud et al., 2012; Peel et al., 2012a)
Enterococcus species	++	40	(Schäfer et al., 2008; Lee et al., 2010; Shukla et al., 2010; Kim et al., 2011; Peel et al., 2012a)
Pseudomonas aeruginosa	+++	212	(Berbari et al., 2010; Lee et al., 2010; Shukla et al., 2010; Kusuma et al., 2011; Mahmud et al., 2012; Peel et al., 2012a)
Escherichia coli	++	247	(Biring et al., 2009; Lee et al., 2010; Kusuma et al., 2011; Mahmud et al., 2012)
Acinetobacter baumannii	+	59	(Kim et al., 2011)
Klebsiella pneumoniae	+	94	(Cano et al., 2020)

BACTERIAL BIOFILM

Biofilm is part of the bacterial lifecycle in PJI (**Figure 1**). Biofilms are composed of an extracellular matrix made from exopolysaccharides, proteins, teichoic acids, lipids, and extracellular DNA (Arciola et al., 2012). Complex communities of bacteria are engulfed in this extracellular matrix. These communities can be mono- or polymicrobial. One of the consequences of biofilm formation during PJI is the formation of a bacterial reservoir that often leads to symptomatic but non-culturable infection, recurrent or persistent infection, or infectious spread via emboli (i.e., part of the biofilm migrates through the blood) (Azeredo and Sutherland, 2008).

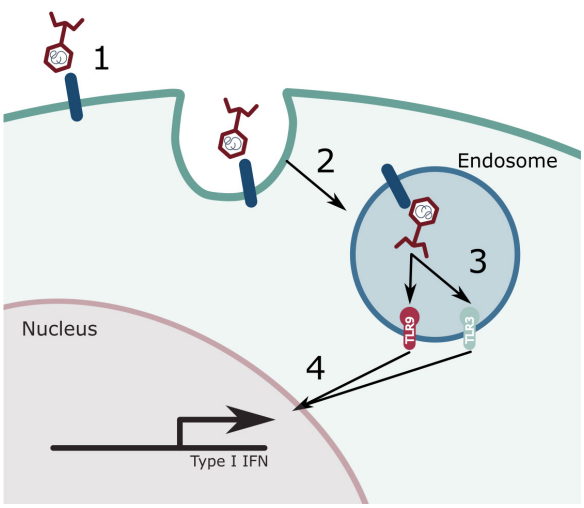

FIGURE 1 | Phage induced immune responses. A variety of immune cells can recognize and interact with phages. (1) Phages are recognized by a currently unknown cell receptor. (2) This leads to the internalization of the phage where it will end up in the endosome. (3) Once present in the endosome, the phage gets degraded and its genetic content can either be recognized by TLR9 (dsDNA) or by TLR3 (dsRNA). (4) The activation of these immune receptors will lead to a signaling cascade and the expression of type I interferon (IFN).

The growth of a biofilm is not static but occurs through multiple stages. Starting with the attachment of the bacterial cell to a surface, followed by the initial growth on the surface, maturation of the biofilm, and finally embolization. In the end, the mature biofilm has a multicellular non-homogeneous structure wherein bacteria communicate with each other through quorum sensing. Quorum sensing make use of chemical signals to help bacteria communicate, coordinate, and cooperate. Quorum is the critical density needed to establish a biofilm colony and express virulence (Miller and Bassler, 2001; Ng and Bassler, 2009). Quorum sensing is a positive feed-forward loop which stimulates population-based gene expression (Seed et al., 1995; Rutherford and Bassler, 2012). Both Gram-negative and Gram-positive bacteria utilize these quorum sensing strategies to facilitate intraspecies communication. Additionally, due to the conserved nature of the quorum signal mechanism, inter-species communication also occurs providing a plausible explanation for cooperative polymicrobial biofilms (Mooney et al., 2018).

Bacterial sub-populations have different functions that ultimately support the whole biofilm. In this biofilm state, bacteria are protected from antimicrobials and the immune system (Donlan and Costerton, 2002). This is partially due to the physical separation of the bacteria from the antimicrobials or the immune cells, but also because bacteria are in a metabolic inactive state, called persistence (del Pozo and Patel, 2007; Molina-Manso et al., 2013). This makes the treatment of PJI with conventional antimicrobials very difficult, mandating surgical intervention, including the removal of the prothesis, to achieve a cure. Some antimicrobial agents have an effect against biofilm-resident bacteria such as rifampicin, but ultimately resistance frequently still occurs (Maudsdotter et al., 2019).

Alternatively, bacteriophages, or their derived proteins can be exploited to treat biofilms.

BACTERIOPHAGE – A BACTERIAL PREDATOR

Phages, viruses that specifically target and infect bacterial cells, can be exploited to treat biofilms in PJI. Phages consist either of DNA or RNA which is encapsulated in a protein coat called a capsid (van Regenmortel, 1992). Bacteriophages are the most abundant biological entity on the world and occur everywhere in the biosphere. They have colonized even such forbidding habitats as volcanic hot springs. Their main habitats are the oceans and terrestrial topsoil (Ackermann, 2011). Phage particles can be tailed, polyhedral, filamentous, or pleiomorphic (Calendar, 2006; Ackermann, 2009). Tailed phages, representing over 96% of all known phage species, constitute the order *Caudovirales* with three families, characterized by contractile (*Myoviridae*), long and non-contractile (*Siphoviridae*), or short and non-contractile (*Podoviridae*) tails (Ackermann, 2011).

The most common phage life cycles are the lytic and lysogenic life cycle. In the lytic life cycle, the phage genome exists within the host but outside the host genome. Lytic or virulent phages repeat a cycle in which self-proliferation is synchronous with the destruction of bacteria (i.e., the lytic cycle or the virulent infection) (Matsuzaki et al., 2005). In this stage, gene expression, genome replication, and morphogenesis occurs (i.e., the formation of the genomes and capsids and the packing of the genomes in the capsids) (Ackermann, 1998). Lysogenic or temperate phages, can remain dormant in the host through integration of its genome in the bacterial genome, called a prophage, replicating along with the host until they are triggered into a lytic lifecycle. For most lysogens this trigger entails DNA damage, which can be triggered by a multitude of stimuli such as antibiotics, reactive oxygen species or UV (Ackermann, 1998; Weinbauer, 2004).

The biological characteristics of phages make them ideal for treating bacterial infections. Their lytic activity, auto-dosing, low inherent toxicity, minimal disruption of normal flora, narrow potential for inducing resistance, lack of cross-resistance with antibiotics, rapid discovery, formulation and application versatility, and biofilm clearance are characteristics looked for in antimicrobials (Loc-Carrillo and Abedon, 2011). Auto-dosing refers to the fact that phages themselves contribute to establishing the bacterial lethal dose by increasing their number during the bacterial-killing process (Carlton, 1999; Skurnik and Strauch, 2006; Chan and Abedon, 2012). A narrow host range limits the number of bacterial types with which selection for specific phage-resistance mechanisms can occur (Hyman and Abedon, 2010). Some phage derived proteins, such as endolysins or depolymerases, are able to degrade the biofilm allowing the phage to destroy the reservoir of bacteria that reside within exopolysaccharide matrix (Hanlon et al., 2001; Tait et al., 2002).

Phages are versatile in terms of formulation and can be combined with antibiotics or incorporated into scaffolds such as hydrogels or wound dressings (Alisky et al., 1998; Kutter et al., 2010; Wroe et al., 2020). They can be applied as liquids, creams, impregnated into solids, in addition to being suitable for most routes of administration (Carlton, 1999; Kutateladze and Adamia, 2010; Kutter et al., 2010). Different phages can be mixed as cocktails to broaden their properties, typically resulting in a collectively greater antibacterial spectrum of activity and lowering the chance of acquiring phage resistant bacterial strains (Merabishvili et al., 2009; Goodridge, 2010).

Phages as pharmaceuticals are protein-based, live-biological agents that can potentially interact with the body's immune system, can actively replicate, and can even evolve during manufacture or use (Loc-Carrillo and Abedon, 2011). Phages possess unique pharmacokinetics and pharmacodynamics that remain poorly understood (Cooper et al., 2016). The pharmacokinetics of phages are complicated due to their self-replicating nature. Critical parameters that affect phage therapy are the phage adsorption rate, burst size (the number of phages released by one infected bacteria), latent period (the time between phage infection and bacterial lysis, i.e., the time needed to assemble new phage progenies), initial phage dose, and density-dependent thresholds (Payne and Jansen, 2001). Another important parameter is the clearance rate of the phage particles from the body fluids by the reticuloendothelial system. Although phages are considered generally well penetrating different tissues and body organs they may significantly differ in bioavailability after oral application (Międzybrodzki et al., 2017b; Dąbrowska, 2019). Their stability in environment is one of the limiting factors for production of standard phage medicinal products which require longer storage (Jault et al., 2018; Jończyk-Matysiak et al., 2019).

PHAGES AND THE IMMUNE SYSTEM

Historically, phages were regarded as immunologically inert. However, phages do cause a humoral immune response (Ochs et al., 1971; Łusiak-Szelachowska et al., 2014; Hodyra-Stefaniak et al., 2015; Majewska et al., 2015; Zaczek et al., 2016). The production of anti-phage antibodies can affect the outcome of phage therapeutic interventions (Łusiak-Szelachowska et al., 2014). Furthermore, the route of administration plays a big role on the level of antibody production (Zelasko et al., 2016; Łusiak-Szelachowska et al., 2017). For instance, oral administration seems to lead to the lowest level of anti-phage antibodies compared to intraperitoneal injection in mouse models (Dąbrowska, 2019). Moreover, low levels of anti-phage antibodies have also been detected in human subjects after oral administration of phage (Międzybrodzki et al., 2017b). Although an antibody response might be present during or after a phage therapeutic intervention, this does not necessarily lead to a reduction of the therapeutic potential (Łusiak-Szelachowska et al., 2014, 2017; Zelasko et al., 2016; Dąbrowska, 2018Dąbrowska, 2019).

However, recent research has demonstrated a phage-induced innate immune response (**Figure 1**). Moreover, mathematical models have predicted their importance in the outcome of a therapeutic intervention (Van Belleghem et al., 2018). As

expected, this phage induced response appears to mimic an antiviral response (Van Belleghem et al., 2017; Gogokhia et al., 2019; Sweere et al., 2019). The antiviral immune response is driven by a Toll-like receptor (TLR) 9 response to *Caudovirales* DNA (Gogokhia et al., 2019) whereas it is driven by a TLR3 response to *Inoviridae* RNA (Sweere et al., 2019). The antiviral immune response may help the phage escape clearance or enable the bacterial host to thrive.

Interestingly, some phages or their preparations may exert anti-inflammatory activity (Van Belleghem et al., 2017, 2018). A significant decrease in C reactive proteins (CRP) was observed in some patients treated with phages, even in the absence of clearing the bacterial infection (Gorski et al., 2016). Moreover, it has been shown that *Escherichia coli* phage T4 presents a strong anti-inflammatory effect in mouse models reflecting the autoimmune reaction corresponding to rheumatoid arthritis (Międzybrodzki et al., 2017a). These observations are in accordance with observations in humans, suggesting that phage therapy may modify the immune responses.

PHAGES AIDING SUPPRESSIVE THERAPY

The minimal inhibitory concentration (MIC) of an antibiotic is determined on cultured bacteria and does not reflect the susceptibility of the bacteria within a biofilm. Killing the bacteria within a biofilm requires a many-fold higher concentration of antibiotic to achieve the minimum biofilm eradication concentration (MBEC) (Ricciardi et al., 2020). Thus, the use of suboptimal antibiotic concentrations could lead to antibiotic resistance in the setting of PJI. Phages are an ideal alternative or adjunct to antibiotics for treating or suppressing PJI (**Table 1**). Phages have a proven track record for combating, and in some cases eradicating biofilms (Tkhilaishvili et al., 2020). Even though bacteria in a biofilm, such as small colony variants, have a decreased cellular metabolic activity that often makes them resistant to antibiotics. Furthermore, studies have shown synergy between the use of systemic antibiotics and phages to treat biofilm-associated infections, although the precise mechanism is currently not known (Yilmaz et al., 2013; Kamal and Dennis, 2015; Torres-Barceló et al., 2016). On the downside, antagonistic effects between phages and antibiotics have been observed as well. *In vitro* antagonism between a mixture of two *P. aeruginosa* phages and high doses of tobramycin (Kamal and Dennis, 2015) was observed in which MBEC tobramycin was effective against *P. aeruginosa* biofilms, but its effect diminished when phage was added. This is in line with an observation that phage may sequester antibiotics, thus lowering the active concentration (Tarafder et al., 2020). Hence, when combining antibiotics and phages, strategies may have to be considered for sequential administration.

Furthermore, the occurrence of phage resistance provides an additional hurdle for the use of phage as a therapeutic (Smith and Huggins, 1983; Levin and Bull, 2004). In the therapeutic setting, these phage resistant strains can occur in 17–86% of treated patients, depending on the pathogen (Międzybrodzki et al., 2012). However, phage resistance often comes at the cost of reduced bacterial virulence and can even be accompanied by re-sensitization to antibiotics (Capparelli et al., 2010; Gu et al., 2012; León and Bastías, 2015; Chan et al., 2016; Oechslin, 2018).

Only a limited amount of pre-clinical studies have evaluated the potential use of phages to treat PJI (Yilmaz et al., 2013; Kaur et al., 2016; Kishor et al., 2016; Ferry et al., 2018a,b; Cano et al., 2020), although attempts to treat PJI-like diseases, such as osteomyelitis, with phage date back to the early 1930s (Albee, 1933). Different administration routes can be deployed, as recently reviewed by Dąbrowska (2019), of which oral administration or injection (intraperitoneal, intramuscular, or subcutaneous) are the most common (Dąbrowska, 2019). The actual dose needed to obtain a therapeutic effect is still debated within the field with reports showing as low as 10^3 pfu/ml being sufficient to eradicate a bacterial infection (Soothill, 1994; Marza, 2006), with general consensus saying a minimum of 10^6 pfu/ml is needed (Morozova et al., 2018).

Phages have been used to treat PJI in the context of antibiotics (Ferry et al., 2018b). For example, in 2018, a patient with relapsing PJI of the right hip was treated by injecting a cocktail of phages into the joint in addition to systemic antibiotics. Eighteen months after phage therapy, the clinical signs of PJI were absent. This case shows the efficacy of phages in a PJI setting, although surgical intervention was still necessary for this treatment and it is unclear whether this represents suppression prior to phage resistance or eradication. Another recent case with a patient with a right total knee arthroplasty 11 years prior, suffering multiple episodes of PJI despite numerous surgeries and prolonged courses of antibiotics, showed progressive clinical worsening and development of severe allergies to antibiotics, had been offered limb amputation for his persistent right prosthetic knee infection due to *Klebsiella pneumoniae* complex. As a last resort he was offered intravenous phage therapy (Cano et al., 2020). The patient received 40 doses of a single phage spread over 8 weeks, in combination with minocycline and was able to circumvent further surgery. Furthermore, the authors were not able to identify any phage resistant strains over this eight-week course of phage treatment. This might be due to the lower metabolic activity of the bacteria in the biofilm leading to a lower chance of phage resistance to occur.

PHAGES PREVENTING PJI

Prophylactic strategies require anticipating a certain bacterial infection in order to provide the necessary agents to combat a not yet existing infection. Nevertheless, additional research is needed to further extend the lifetime of these phages after they undergo the coating or impregnation strategies to provide long lasting protection (**Figure 2**). It is currently not well described what amount of phage inactivation could be expected or is accepted when mixing phages with bone cement or coating them on prosthetic surfaces. Also getting a clear view of the commensal flora of a patient will become valuable in order to make educated guesses as to which phages to prevent PJI. The main difficulty with using phages in this manner is that they will lose their activity after one round of infection. This enables the removal of

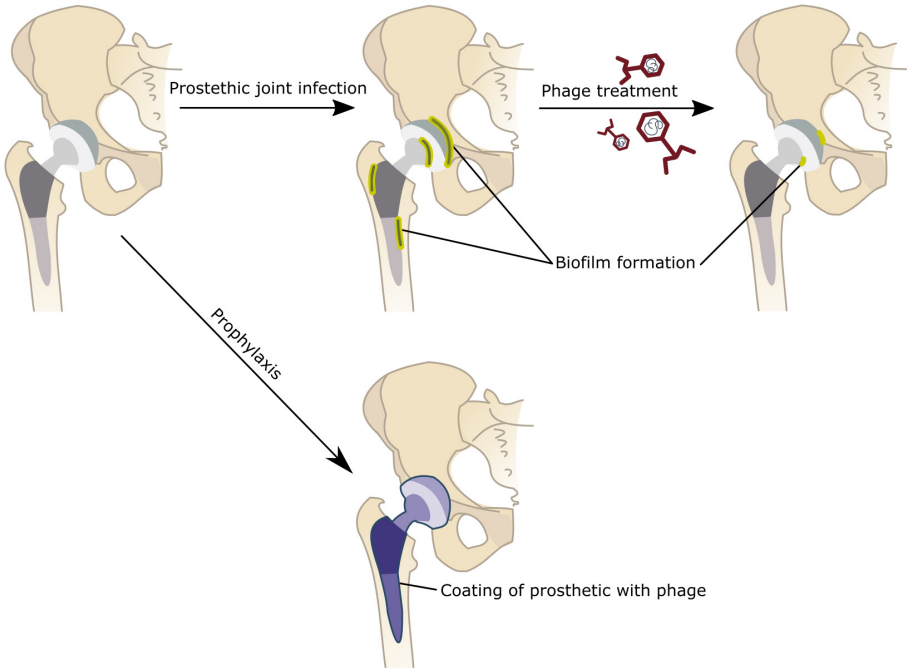

FIGURE 2 | Alternative strategies for treating or preventing Prosthetic joint infections (PJI). Although that joint replacement is a life-enhancing procedure, an unfortunate minority experiences pain and will ultimately require additional surgery. Septic failure, i.e., periprosthetic joint infections, are the most common reason for joint replacement failure. During PJI, bacteria bound to the prosthetic will form biofilm structure that become resistant to antibiotic treatments. Hence the treatment of PJI requires the removal of these biofilm contaminated implants in addition to the administration of antibiotics. The use of phage can form a valid alternative (or additive to classic antibiotic treatments) to treat these PJIs without the need of a surgical intervention. These phages can be administered orally as a liquid or in a powdered formulation or injected intravenously or as a hydrogel directly in the joint. Alternatively, phages can be used to prevent the occurrence of PJI by either mixing phages in the bone cement or coating the implant with phages. In case of coating implants with phage, one could opt for a single phage or a cocktail of phages. A gradual release system could be applied, using hydroxypropyl methylcellulose (HPMC) to gradually release and deliver the therapeutic agents at the potential sites of infections. This might prevent the establishment of biofilms and the occurrence of PJIs.

an initial infection but would not enable the clearance of a future recurrent infection. To tackle this problem the bioavailability of the phage can be altered by embedding them in a matrix enabling the slow release from the prosthetic bone cement over time and in different waves.

After the selective identification, patients could be decolonized of offending organisms prior to surgery, reducing surgical site infection and PJI after joint arthroplasty. The downside of the use of antibiotics for decolonization, especially in PJI, is the occurrence of antibiotic resistant strains. However, this problem would not arise when phages are used to disinfect the site of surgery. The use of phage would remove the targeted bacteria without disturbing the commensal flora or inducing dysbiosis in the patients gut or skin.

Other approaches that could be used is to directly interfere with the biofilm formation. Research has focused on disrupting biofilm formation by interfering with the quorum sensing (Sully et al., 2014; Atwood et al., 2016; Grandclément et al., 2016). These compounds target a variety of steps in the quorum sensing pathway, including the inhibition of quorum sensing signal production through degradation or substitution of SAM or acyl-ACP (precursors to acyl-homoserine lactone). Sequestration of quorum sensing signals using antibodies have also been evaluated as a potential strategy (Park et al., 2007). Alternative strategies have looked to impair the quorum signal transduction through the disruption kinase domain involved in the quorum sensing transduction (Atwood et al., 2016; Grandclément et al., 2016). Again phages could play a potential role in preventing the formation of biofilms, not due to their direct lytic activity but due to the potential effects of depolymerases present on certain phage tails (Azeredo and Sutherland, 2008; Knecht et al., 2020). These depolymerases could help degrade the biofilm matrix enabling the immune system to more effectively clear a starting bacterial infection.

The optimal form of delivery of phages to the joint implant site is unclear. Phages can be impregnated into bone cement, polymethyl methacrylate (Samokhin et al., 2018). However, once phages are impregnated into polymethyl methacrylate they lose their effective titer between over 1–2 weeks.

Recent research points into the potential of using phages to coat prosthetic materials. Different strategies could be applied, one could coat with a single phage or a phage cocktail either to one specific pathogen or a diverse set of pathogens. Kaur et al. (2016), showed the potential of using phage and antibody coated Kirshner wire to prevent *S. aureus* infection when used at the site of the prosthetic (Kaur et al., 2016). The authors used a hydroxypropyl methylcellulose (HPMC) gel, for the gradual release and delivery of two therapeutic agents at the implant

site. These coatings of the Kirshner wires remained stable over 20 days, although a 3-log reduction could be observed after initial coating. Moreover, the authors observed that the elution from this gel remained steady for 48 h. A combinatorial approach of the *S. aureus* phage and linezolid led to a reduction of bacterial adhesion by 4-log, as well as reducing the occurrence of phage resistant strains when phage alone was used (Kaur et al., 2016).

CONCLUSION

Orthopedic devices are prevalent and durable making them one of the most common surgical implant types to become infected (Inzana et al., 2016). All of the materials used for implantable orthopedic devices are easily colonized by bacteria (Gbejuade et al., 2015). Nevertheless, several preventative and therapeutic strategies exist, some more invasive then others. Bacteriophage is a valuable addition as the field looks to control antimicrobial infection in a more effective manner – moving beyond the morbidity of the scalpel and delivering higher doses of resistance-generating antibiotics. A very promising path is the use of phage coated prosthetics. Although the pitfall here lies in the fact that the immobilized phage will lose its activity after one round of infection. This would still allow to combat an initial infection but not a recurrent one. To tackle this problem the bioavailability of the phage can be altered by embedding them in a matrix enabling the slow release from the prosthetic bone cement over time and in different waves. Alternatively, phage embedded in hydrogels and injected directly at the site of infection could be performed on patients that already have an implant to remove the infection. This would enable to physicians to treat PJIs without the need of surgical intervention or removal of the implant, providing layover between the development of phage coated prosthetics and the use of phage in PJI. The use of phages could also enable to combat current unculturable bacteria, on the condition that they can easily be identified through genomic approach. It has been suggested that machine learning approaches can be utilized to either identify, or generate through synthetic genomics, based on the genomic information provided on the bacterial target (Leite et al., 2018; Martorell-Marugán et al., 2019; Baláž et al., 2020; Pirnay, 2020).

Nevertheless, to use phages under these circumstances the field needs to further invest in understanding the bioavailability and biodistribution of phages as well as their immunogenicity in order to generate the best outcome for the patients. Although rigorous clinical trials are currently lacking progress has begun to treat PJI with phage.

AUTHOR CONTRIBUTIONS

All authors participated in the conception, drafting, and/or editing of the manuscript.

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Overcoming Challenges to Make Bacteriophage Therapy Standard Clinical Treatment Practice for Cystic Fibrosis

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Individuals with cystic fibrosis (CF) are given antimicrobials as prophylaxis against bacterial lung infection, which contributes to the growing emergence of multidrug resistant (MDR) pathogens isolated. Pathogens such as *Pseudomonas aeruginosa* that are commonly isolated from individuals with CF are armed with an arsenal of protective and virulence mechanisms, complicating eradication and treatment strategies. While translation of phage therapy into standard care for CF has been explored, challenges such as the lack of an appropriate animal model demonstrating safety *in vivo* exist. In this review, we have discussed and provided some insights in the use of primary airway epithelial cells to represent the mucoenvironment of the CF lungs to demonstrate safety and efficacy of phage therapy. The combination of phage therapy and antimicrobials is gaining attention and has the potential to delay the onset of MDR infections. It is evident that efforts to translate phage therapy into standard clinical practice have gained traction in the past 5 years. Ultimately, collaboration, transparency in data publications and standardized policies are needed for clinical translation.

Keywords: bacteriophage, cystic fibrosis, lung disease, alternative therapy, animal models, antimicrobials, biofilms, regulation

INTRODUCTION

Cystic Fibrosis (CF) is a life-limiting genetic disease caused by mutations to the Cystic Fibrosis Transmembrane Regulator (CFTR) gene. There are 2,000 variant mutations in the CFTR gene, however, more than 70% of people with CF carry the p.Phe508del mutation. Currently there are six classes of mutations, each with varying degrees of disruption to CFTR protein production and function (Pettit and Fellner, 2014; Lopes-Pacheco, 2016; Veit et al., 2016), correlating to varying severity of disease phenotype. The genetic defect leads to impaired water and electrolyte traffic of airway epithelial cells (AECs), tenacious airway surface liquid, impaired mucociliary clearance

mucus build-up in the lungs of those afflicted due to the inability of AECs to allow chloride ions to pass into the airway surface liquid (Crawford et al., 1991; Haq et al., 2016). This leads to airway obstruction, accelerated lung function decline, reduced quality of life, and ultimately premature lung failure and death. With defective mucociliary clearance, accumulation of mucus in the airway of CF lungs occurs, creating an ideal microenvironment for the growth and persistence of bacteria. The inability to clear these bacteria allows opportunistic pathogens to establish a niche within the environment, ensuring their survival (Moreau-Marquis et al., 2008; Bjarnsholt et al., 2009; Ramsay et al., 2016).

With an increasing lifespan of CF patients due to currently available therapies and surveillance programs, isolation of multidrug resistant (MDR) bacteria from the respiratory tract has also increased. *Pseudomonas aeruginosa* (*P. aeruginosa*) remains by far the most common airway pathogen particularly amongst adults with CF. The CF Foundation has recently reported ∼17.9% of *P. aeruginosa* isolated from CF individuals in North America were MDR (Cystic Fibrosis Foundation, 2019). An earlier study conducted across CF centers in Australia found that 31 and 35% of *P. aeruginosa* isolated from a pediatric and an adult cohort, respectively, were also MDR (Smith et al., 2016). It is known that the lungs of CF individuals are prone to bacterial colonization, particularly by *P. aeruginosa*. Once colonized, the bacteria are impossible to eradicate and individuals undergo long-term antimicrobial regimes to treat and prophylactically control the rate of infection. The increasing rates of MDR infections are attributed to the prolonged use of antimicrobial drugs for both treatment and prophylaxis, and consequent gain of resistance genes and selection of hypermutator isolates (Kidd et al., 2013, 2018). Cross infection of MDR *P. aeruginosa* strains due to less stringent infection control measures had also contributed to the rapid rise in MDR rates over the past two decades (Salunkhe et al., 2005; Johansen et al., 2008; Fothergill et al., 2012; Parkins et al., 2014). Treatments for MDR pathogens are accompanied with a caveat; cumulative antibiotic burden leads to development of drug allergy and toxicity (Levison and Levison, 2009; Kalghatgi et al., 2013; Gao et al., 2017). Progressive limitation in antimicrobial treatment in CF, particularly in the aging population given the emergence of MDR organisms, antimicrobial allergy and toxicity are associated with lengthier hospitalizations, increased rates of re-admittance, and extensive treatment regimens (Baumann et al., 2003; Sansgiry et al., 2012).

The issue of MDR is now so widespread that World Health Organization (WHO) has declared the issue of antimicrobial resistance a global crisis (Shrivastava et al., 2017). The discovery pipeline into new classes of antimicrobials is also slow since it is relatively unprofitable, and innovations are unable to keep up with emerging resistance. To address this, alternative treatment methods must be explored. Treatment strategies targeting bacterial virulence and resistance have been studied extensively. Anti-virulence compounds such as quorum sensing inhibitors (Müh et al., 2006; Brackman et al., 2011; O'Loughlin et al., 2013; Soukarieh et al., 2020) and iron chelation (Moreau-Marquis et al., 2009; Parquet et al., 2018) have been found to be successful in inhibiting biofilm formation, reducing pathogenicity and increasing susceptibility to traditional antimicrobials. Strategies targeting resistance have included investigating efflux pump inhibitors (Sabatini et al., 2013; Shriram et al., 2018), anti-sense oligomers (Geller et al., 2013; Sawyer et al., 2013), immunotherapy (Feigman et al., 2018), host defense peptides (Overhage et al., 2008; de la Fuente-Núñez et al., 2015) and bacteriophages. Many of these strategies are still in exploration and validation phases and are still some way off from translating to standard clinical care practice. The vital need for a swift translation of alternative therapy into clinical use has identified bacteriophage (phage) therapy as one of the top candidates due to its successful use in humans when approved on compassionate grounds. Phage treatments are also cheaper due to shorter treatment periods, exhibit little or no toxicity, and are more effective than current antimicrobial strategies (Alemayehu et al., 2012; Agarwal et al., 2018; Oliveira et al., 2020).

BACTERIOPHAGES AND THEIR THERAPEUTIC APPLICATIONS TO DATE

Bacteriophages are viruses found ubiquitously on Earth. They are able to undergo two life cycles: lytic (lyse the bacterial host in the process of replication) or lysogenic (integrate into the genome of bacterial host). Thus, when considering these for therapeutic application, selection should primarily be limited to the use of lytic phages in order to minimize the possibility of virulence or resistance genes transfer. They were first described and observed to display lytic activity in the early 1910s by microbiologists Frederick W. Twort and Felix d'Herelle (Twort, 1914; d'Herelle, 1917) but despite their initial success, they were soon overshadowed by the introduction of antibiotics (Clokie et al., 2011). Antibiotics were considered a cheap and safe way to treat bacterial infections and their assessment was typically accompanied with well documented research that demonstrated their beneficial effects. Although resistance against penicillin emerged almost immediately after its introduction, discovery of new antibiotics maintained their primary use in the Western world. With restricted access, eastern bloc countries progressed phage therapy through to its clinical inauguration. Entities including the Eliava Institute of Bacteriophages, Microbiology and Virology (EIBMV) in Georgia and the Phage Therapy Unit at the Hirszfeld Institute of Immunology and Experimental Therapy in Poland are still operational and patients are provided personalized phage therapy for chronic infections (d'Herelle, 1931; Häusler, 2006; Kutateladze and Adamia, 2008; Chanishvili, 2012, 2016; Kutateladze, 2015; Górski et al., 2018).

In the West, hesitancy in translating to a human treatment pipeline has been primarily due to the lack of published scientific reports and rigorous safety data. However, human phage therapy has gained significant traction in the last 5 years and has been tested clinically (**Figure 1**). One study used phage therapy to treat a patient who had been infected with MDR *Acinetobacter baumanii* (*A. baumanii*) (Schooley et al., 2017)

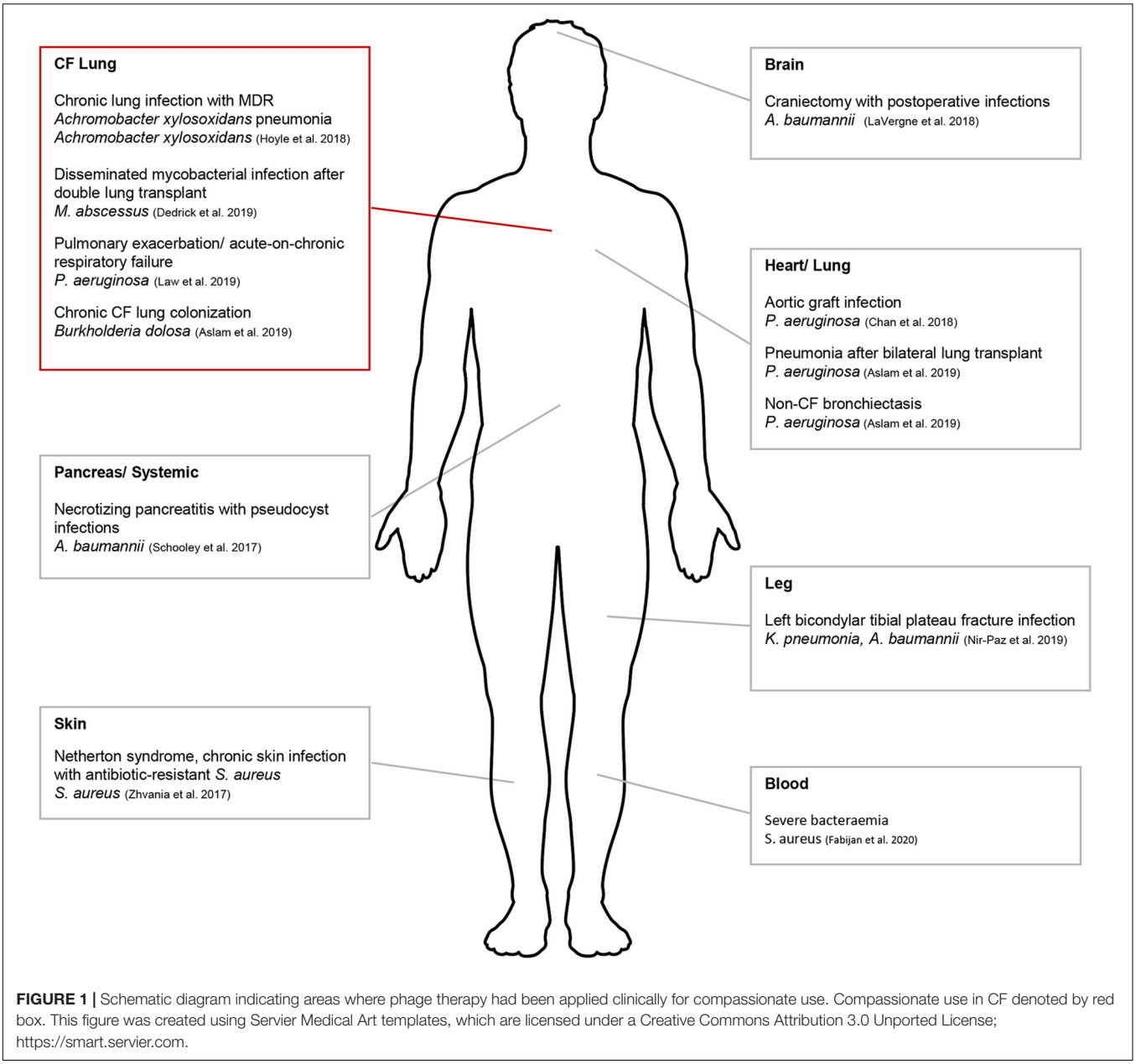

FIGURE 1 | Schematic diagram indicating areas where phage therapy had been applied clinically for compassionate use. Compassionate use in CF denoted by red box. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

(**Figure 1**). Multiple rounds of antibiotic treatments had initially failed to control the infection which had spread beyond the abdominal cavity. *In vitro* experiments were initially conducted to determine phage specificity and efficacy against the infective strain and approval for clinical use was then sought from the Food and Drug Administration (FDA). This was granted on compassionate grounds and treatment resulted in the patient's full recovery (Schooley et al., 2017). Since this pioneering study, compassionate ground use of phage therapy has been successfully used to treat patients with distinct MDR infections (Chan et al., 2018; Dedrick et al., 2019). A recent Australian study has highlighted the translatable potential of phage therapy into clinical applications to treat multiple patients suffering from severe *Staphylococcus aureus* (*S. aureus*) bacteremia (Petrovic Fabijan et al., 2020b). While this specific study supported phage therapy for disseminated bacterial infection, what is less clear is whether it is applicable for patients suffering from chronic soft tissue infection caused by biofilm-producing bacteria.

THE POTENTIAL OF PHAGE THERAPY IN CF

In order to more accurately assess how phage therapy is currently advancing, we conducted an initial review of articles in NCBI using the article title search term "Bacteriophage." We selectively excluded all published abstracts and still identified >12,000

published articles in this area. When "Therapy" was included in the article title search term, 141 published articles were found, including 46 review papers. The remaining 95 original research papers were then screened and found to report on research performed in the medical (83) or veterinary sphere (12). Collectively, these findings imply that despite an enormous amount of research being carried out in the discovery and characterization of phages, the field has struggled to translate results into standard clinical care. Although successful use of phage therapy in CF has been reported, this has been approved on compassionate grounds and its translation into standard clinical care still requires additional rigorous, systematic and detailed exploration (**Supplementary Table 1**).

While *P. aeruginosa*, *S. aureus*, and *Burkholderia cepacia* complex (*B. cepacia* complex) are commonly isolated pathogens from individuals with CF, *Mycobacterium* and other infections are emerging. One ideal therapeutic target is *Mycobacterium abscessus* (*Mab*), an emerging pathogen isolated from the lungs of individuals with CF (Bernut et al., 2019). *Mab* is able to form biofilms (Qvist et al., 2015; Fennelly et al., 2016) and is intrinsically resistant to many antitubercular drugs, requiring prolong usage of at least three antimicrobial drugs for up to 2 years (Floto et al., 2016). While there has been clinical evidence correlating *Mab* infections with declining lung function, whether the bacterium is the causative agent is unknown (Sanguinetti et al., 2001; Esther et al., 2010; Qvist et al., 2016). The first reported compassionate use of phage therapy in CF was for a 15-year old patient with disseminated *Mab* infection at sites other than the lung following bilateral lung transplant (Dedrick et al., 2019; **Figure 1**). Despite additional antimicrobials used, *Mab* had infected other areas of the body apart from the surgical site. A cocktail of three phages was used and were bioengineered from mutant derivatives that displayed optimum lysis of *Mab* isolated directly from the patient. A single topical application was followed by intravenous therapy for ∼32 weeks. Upon completion, lung function had improved from 50% FEV_1, immediately following transplant up to 80–90% FEV_1. The significance of this study lies in the outlined pipeline needed to ensure phage therapy translation including appropriate *in vitro* safety and efficacy studies.

The second reported compassionate use of phage therapy in CF was for a 26-year old patient who presented with severe acute-on-chronic respiratory failure resulting in mechanical ventilation (Law et al., 2019). The patient was infected with two strains of MDR *P. aeruginosa* and received multiple courses of high dose antibiotics which then triggered kidney failure. Approval for AP-PA01, a cocktail of four bacteriophages produced by AmpliPhi Biosciences Corporation (now Armata Pharmaceuticals) was granted and phages were administered intravenously. After 8-weeks of treatment, the patient was successfully cleared of *P. aeruginosa* colonization without side effect, was ambulatory and stable enough to be once again placed on a lung transplantation waitlist. Other pathogens targeted by phage therapy in CF have included *Achromobacter xylosoxidans* and *Burkholderia dolosa* (**Figure 1**), due to increasing incidences of multidrug resistance (Kalish et al., 2006; Jeukens et al., 2017). Phage therapy has also been applied for compassionate use at various sites other than the lung (non-CF associated) suggesting that a number of other CF-associated pathogens including *S. aureus*, *A. baumanii*, and *Klebsiella pneumoniae* may also be clinically targeted using phage therapy (**Figure 1**). Emerging CF pathogens such as *Stenotrophomonas maltophilia*, are also of concern due to their increasing incidence of isolation (Millar et al., 2009; Razvi et al., 2009; Emerson et al., 2010) and multidrug resistance (Gajdács and Urbán, 2019; Gröschel et al., 2020). Since their infectivity and transmission mechanisms are still to be elucidated (Stanojevic et al., 2013; Gröschel et al., 2020) which would typically direct targeted treatment regimens, phage therapy has been postulated as a potential therapeutic option currently (Chang et al., 2005; Peters et al., 2015, 2017).

There have also been attempts to assess phage therapy as part of potential standard clinical care, however, rarely have results of performed clinical trials been published. It is critical to report this information in order to inform the trial process and guide future study designs. An example identified through a screen of registered trials on ClinicalTrials.gov is "MUCOPHAGES" (NCT01818206). Although identified as completed, there is no available information on formulation used, length of phage exposure, and importantly what bacteria were being targeted. Closed findings only act to hinder progress and there needs to be greater transparency if we are to pipeline this therapy into standard clinical care.

Another challenge is that most published studies on the efficacy of phage therapy on CF-derived pathogens have been performed using pathogens in planktonic state. However, bacterial pathogens often exist in biofilm state within the CF airways. Such significant differences between the *in vitro* experiment model and *in vivo* CF airway conditions render the translation of benchside result to bedside questionable. Biofilms are made up by a pure population or a consortium of microorganisms, creating a unique bacterial lifestyle and niche habitat, enhancing protection against antimicrobials, and able to establish in conditions with a flow of liquid and withstand shear force, forming classic tower-like structures (Flemming and Wingender, 2010; Flemming et al., 2016). Thus, phages with effective biofilm dispersal capabilities are highly desirable. Current evaluation of phage activity on biofilm is usually performed on abiotic surfaces which typically do not account for biological flow strength, nor the properties of the tissues where typically biofilms reside, namely (in the case of CF) the airway epithelium. Flow-cell systems and fluorescence microscopy are considered the "gold standard" to observe spatiotemporal changes of biofilm heterogeneity in real-time (Crusz et al., 2012; Tolker-Nielsen and Sternberg, 2014; Haagensen et al., 2015). The use of flow-cell also maintains biofilm's viability while the hydrodyanamic movement removes planktonic bacteria and eliminates a confounding factor in the measurement of phage therapy efficacy on the biofilms. Thus, having a biological relevant model of CF would assist in our understanding not only of biofilm formation but also of bacterial evasion of the host innate and adaptive immune responses.

Phage Therapy: CF Experimental Models and What Is Missing

While lung disease is the major cause of morbidity and mortality in CF, there are currently no effective models that mimic the CF lung infection pathology (**Table 1**). Despite different animal models developed to study CF, the data generated often are still limited by the models. The CF porcine model successfully mimics several CF manifestations including meconium ileus (MI), pancreatic deficiency and subsequent gastrointestinal tract obstruction with similar airway epithelia and submucosal gland (SMG) activities at birth to humans (Rogers et al., 2008). However, the model is not commonly used due to severe disease pathology and difficulties in maintaining longevity of the pigs due to MI. High animal husbandry costs also make the model unfeasible for most laboratories to utilize. CF murine and rodent models have also been explored to mimic the pathophysiological states of CF *in vivo*. The CF mouse model was developed shortly after the discovery of the CFTR gene (Snouwaert et al., 1992) where it successfully mimicked chronic lung infection with mucoid strains of *P. aeruginosa* (Coleman et al., 2003). However, differences between the abundance and distribution of airway epithelial cell (AEC) types make the model less translatable to the human CF airway (Plopper et al., 1983; Grubb and Boucher, 1999). In comparison, rats have more developed SMGs and their implication in CF lung pathology is well studied and linked with disease progression in humans (Smolich et al., 1978; Jayaraman et al., 2001; Wine, 2004; Tuggle et al., 2014). Mucus plugging, one of the characteristics of CF lung disease has been observed in the lungs of a $Cftr^{-/-}$ rat model developed by Birket et al. (2018). This is an essential requirement when studying mucociliary transport and bacterial colonization (Birket et al., 2018). Chronic infections are also difficult to establish in these models and typically use bacterial cells embedded into agar beads before lung installation (Bragonzi et al., 2009; Facchini et al., 2014; Kukavica-Ibrulj et al., 2014; Kukavica-Ibrulj and Levesque, 2015; Bayes et al., 2016; Cigana et al., 2016, 2020). Results are also hard to interpret since it is difficult to distinguish induced immune response initiated by bacteria such as *P. aeruginosa* and the presence of a foreign body (agar beads). Finally, the $Cftr^{-/-}$ ferret model resulted due to the high similarity in the anatomy and biology of its lungs with those of humans (Sun et al., 2008). The CF ferret model mimics the human condition in its susceptibility to lung infection, and lung function decline as the main cause of mortality (Sun et al., 2010, 2014). Despite this model being the best to test therapeutics against lung infection and inflammation, progress in this field remains slow as inconsistencies in severity of lung disease confound the ability to understand the impact of disease progression and efficacy of therapeutics.

Cell Cultures and the Role They Play in Phage Therapy Research

An alternative to the use of animal models is the use of AEC cultures. Universal cell lines such as H441 (lung epithelial cells) (Hermanns et al., 2004) and HeLa (cervical carcinoma cells) (Scherer et al., 1953; Lucey et al., 2009) have been utilized in a wide variety of research ranging from cancer to infection models. Immortalized CF cell lines such as CFBE45o – (CF bronchial epithelial cells) (Gruenert et al., 2004) and CuFi (Zabner et al., 2003) have also been instrumental in different studies including the development and efficacy testing of therapies, both genetic and pharmacological. AEC lines have also been valuable in high-throughput screening of potential drugs. However, submerged monolayer cultures of CF AEC lines are unable to mimic the microenvironment of a CF lung, which is characterized by mucus production and persistent inflammation status. Another limitation of CF AEC lines is the inability to test for interactions and efficacy of phage therapy when used in conjunction with CFTR modulators that target specific mutations in the CFTR gene. However, the use of primary AECs obtained from patients would be able to overcome this limitation. Although CF AEC lines can be grown at air-liquid interface (ALI), there have been inconsistencies in barrier integrity, as well as their capacity to form the multiple cell layers that comprise the airway lining architecture. Furthermore, it is unknown whether they also produce multiple cell types typically including goblet cells (Hermanns et al., 2004; Ren et al., 2016).

Fully differentiated primary AECs have been described extensively in the literature and serve as the most representative *in vitro* model of the airway (Randell et al., 2011; Martinovich et al., 2017). Although AEC ALI cultures may not mimic the biological environment perfectly, they demonstrate features of the airway, with polarization of progenitor cells into ciliated, basal, undifferentiated columnar and secretory cells. Tight junctions are also well developed in 3D ALI cultures (Crystal et al., 2008; Choi et al., 2016; Looi et al., 2016, 2018). A concerted effort by the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) to biobank AECs collected from children with CF longitudinally has created a valuable repository that is readily accessible (Sutanto et al., 2011; Garratt et al., 2017). With prospective sampling, an *in vitro* AEC ALI model could be built in the laboratory to screen for and test efficacies of phages against clinical isolates from CF patients, accounting for immunological responses arising from the application of phages. Currently, there are no appropriate biofilm models grown on AECs described in the literature at the time of writing this review (2020). While most research on medical biofilms has been carried out on abiotic surfaces (Alemayehu et al., 2012; Tinoco et al., 2016) or submerged monolayers of AEC (Trend et al., 2018), these are not reflective biological representations of the complex airway epithelium where biofilms establish. Despite advances in cell culture methodologies, recapitulating the entire human lungs remains a challenge. The human lung is a complex organ with more than 59 cell types located in different anatomical locations (Travaglini et al., 2019) and compounding this further, is the complex genotypic and molecular mechanisms within and between these cells. However, a laboratory model is still essential for airway phage therapy application and AEC ALI cultures still remain the most relevant model that mimics key features of the airway epithelium. Recent developments in 3D printing of organs is rapidly evolving and may be able to add further complexity whilst remaining accurate in its recapitulation of the human lung (Grigoryan et al., 2019; Shrestha et al., 2019).

TABLE 1 | Comparison of available CF animal models.

Animal	Advantages	Disadvantages
Pig	• High genetic and anatomic similarity of organs (Cooper et al., 2008; Längin et al., 2018; Sykes and Sachs, 2019).	• Expensive husbandry (Ostedgaard et al., 2011).
	• Highly similar disease pathology with humans (Meyerholz et al., 2010).	• Requires large animal facility.
	• Comparable immunological response to infections.	• Deaths soon after birth due to meconium ileum (MI) (Rogers et al., 2008).
Mouse	• Chronic lung infection with mucoid strains of *P. aeruginosa* (Coleman et al., 2003).	• Submucosal gland (SMG) present only in proximal trachea (Colledge et al., 1995; Hikke Van Doorninck et al., 1995; Zeiher et al., 1995).
	• Similar lung physiological structure after infection.	• Do not display early CF lung phenotype
		• Less severe disease pathology in the lungs
		• Inconsistencies in animal husbandry, strains of *P. aeruginosa* used (Morissette et al., 1995; Coleman et al., 2003; Bragonzi et al., 2005; Hoffmann et al., 2005).
		• Host response mechanisms different from human CF airway epithelia.
Rat	• Extensive SMGs in airways (Smolich et al., 1978; Jayaraman et al., 2001; Wine, 2004; Tuggle et al., 2014).	• Longitudinal study of KO rats required.
	• Cost-efficient husbandry.	• Stability of *Cftr* gene deletion in rats not reported.
	• Shorter gestation period.	• Fairly new model which requires more investigation toward lung infection with bacteria.
	• Mucus plugging observed, impaired mucociliary transport (Birket et al., 2018).	• Airway disease phenotype yet to be elucidated.
Ferret	• High similarity to human lungs both anatomically and biologically (Darnell et al., 2007).	• Varying degrees of spontaneous lung disease.
	• Extensive SMGs (Smolich et al., 1978; Engelhardt et al., 1992; Sehgal et al., 1996; Wine, 2004).	• Difficult to track lung disease progression (Sun et al., 2010, 2014).
	• Exhibits characteristic responses of human CF lungs to bacterial infections.	• No consistent predominant bacteria isolated from lung microbiome (influenced by the gut microbiome).
	• Shorter gestation time (4–6 months) in comparison to the porcine model.	• Lack of sodium epithelial channel (ENaC) dysregulation.
	• Less costly animal husbandry and smaller animal facility required.	• More expensive animal husbandry than rodent model, limiting wide availability.
Zebrafish	• Low expense maintenance.	• Lacks mammalian organs.
	• Fluorescence tracking *in vivo* (Cafora et al., 2019).	
	• High reproduction rate.	

Delivery of prepared phages could also be tested on AEC ALI cultures to capture a more comprehensive pre and post application reaction of the host mammalian cells (Buckley et al., 2018). While topical and/or intravenous applications of delivery have been effective, they are not ideal for pulmonary infections, particularly in pediatrics. Compliance toward the therapy is an important factor that would ensure optimum efficiency of released phages in the lungs against the pathogens. Therefore, inhalation of phages would be the most suitable for pulmonary infections (Sahota et al., 2015; Malik et al., 2017; Wallin et al., 2019). Currently, studies measuring the stability and efficacy of both liquid (Carrigy et al., 2017) and dry powder formulations (Chang et al., 2017, 2020) of nebulized phages have been conducted and show potential in ensuring the dispersion into lower airway of the lungs. Further work is needed to fully elucidate the best formulation and delivery methodologies before phage therapy can be implemented as part of standard clinical care.

PHAGES AND ANTIMICROBIALS: SAFETY AND THE PARTNERSHIP POTENTIAL

Safety and side effects of phage therapy remain the greatest concerns in the translation to clinical care. To date, there have been no known adverse effects or mild effects that failed to resolve by the end of the treatment reported from the application of phages (Vandenheuvel et al., 2015; **Supplementary Table 1**). Although there have been clinical trials that had been terminated, this was due to a lack of significant improvement (Sarker et al., 2016) or insufficient efficacy (Jault et al., 2019). While animal models do not fully mimic the human's immunological response, Trend et al. (2018) have demonstrated that the application of *P. aeruginosa*-specific phages on primary AECs did not elicit an immunological response (Trend et al., 2018). Furthermore, although Żaczek and colleagues reported that anti-staphylococcal phages applied orally or locally did induce a humoral response in some patients, there was no increase in inflammatory markers or reduction in effectiveness of phages (Żaczek et al., 2016). Safety and tolerability have also been demonstrated by two recent studies using phage therapy to treat *S. aureus* chronic rhinosinusitis (Ooi et al., 2019) and severe sepsis (Petrovic Fabijan et al., 2020b), respectively. In cases of phage therapy directed against *P. aeruginosa* respiratory infections, no adverse phage therapy-related effects have yet been identified (Aslam et al., 2019; Law et al., 2019). Nevertheless, interpretation of potential safety and side effects of phage therapy has been limited by the lack of published results.

Currently, approval for phage therapy in CF has been granted on compassionate grounds. However, translation of phage therapy to standard clinical care would most likely target a larger population of patients infected with antibiotic susceptible pathogens. Those on prophylaxis and prolonged treatment regimes would benefit the most since *P. aeruginosa* isolated from early CF lungs of children have been found to be more similar to environmental strains and susceptible to antimicrobial treatments (Burns et al., 2001; Jelsbak et al., 2007; Marvig et al., 2015). A combination of phage therapy may act synergistically with antimicrobials to potentiate the reduction or delay of MDR infection occurrence (Knezevic and Aleksic Sabo, 2019; Petrovic Fabijan et al., 2020a). Phage therapy in combination with suboptimal concentrations of antibiotics has shown potential in eradicating infection more efficiently, lowering the risk of adverse effects from long-term usage of antibiotics (Kirby, 2012; Knezevic et al., 2013; Kamal and Dennis, 2015). A study by Chan et al. (2016) demonstrated that the application of φOMKO1 was able to restore antibiotic sensitivity in *P. aeruginosa*, which reinforces the potential of phages against drug-resistance (Chan et al., 2016). A further benefit of joint use of antibiotics and phage therapy may be a reduction in the development of bacterial resistance to phages, as even when phage mixtures (cocktails) are employed (Wright et al., 2019), gain of phage resistance can occur throughout the length of therapy (Schooley et al., 2017). Further examples of this approach have recently been reviewed by Tagliaferri and colleagues (Tagliaferri et al., 2019). The potential of phage therapy when used in combination with antimicrobials is yet to be fully exploited and future detailed exploration in this area is warranted.

PHAGE THERAPY: FUTURE PROSPECTS

The future of phage therapy is not necessarily to replace current therapies, rather there is potential for clinical applications to supplement and provide an alternative treatment for infections. With a predicted shift into personalized phage therapy in the immediate future, research in this area is likely to grow at an exponential rate (Pirnay, 2020). However, the full potential of phage therapy can only be achieved when there is transparency and a willingness to share knowledge as well as resources. Ideally, phage libraries should be freely available through a network of collaboration, and information on preparation and delivery methods for phages meant for clinical usage should be well documented. Phage formulation and delivery are also critical considerations in order to direct activity to targeted areas and maximize efficacy. In fact, use of phage therapy already appears to be coordinated in various countries according to national regulations, and by major public health institutes such as Therapeutic Goods Administration (TGA) (Australia) (Donovan, 2017; Lin et al., 2019), Food and Drug Authority (FDA) (United States of America) (Jarow et al., 2017; Puthumana et al., 2018) and the European Medicines Agency (EMA) (Europe) (Balasubramanian et al., 2016; Debarbieux et al., 2016). Importantly, a universal code of ethics should be established and regulatory bodies reach a consensus on the exchange of information, usage of phages as treatment and reporting of treatment outcomes (Furfaro et al., 2018; Borysowski et al., 2019). Due to the critical nature of the rise of MDR, increasing the urgency for phage therapy to be implemented as standard care, alternative therapies to be translated into clinical applications need to be expedited. A concerted effort with both local and

global partners could see phage therapy being translated into standard care in the next 5 years.

AUTHOR CONTRIBUTIONS

RN and AK conceived the review topic and focus. RN conducted literature review and drafted the manuscript. AK, AT, BC, and SS contributed to the structure and content, provided critical review and approved the final version to be published. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.593988/full#supplementary-material

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Development of a Novel Chimeric Endolysin, Lys109 With Enhanced Lytic Activity Against *Staphylococcus aureus*

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As the incidence of antibiotic-resistant bacteria has become increased, phage endolysins are believed as one of the promising alternatives to antibiotics. However, the discovery of potent endolysin is still challenging because it is labor intensive and difficult to obtain a soluble form with high lytic activity. In this respect, the modular structures of Gram-positive endolysins can provide an opportunity to develop novel endolysins by domain rearrangement. In this study, a random domain swapping library of four different endolysins from phages infecting *Staphylococcus aureus* was constructed and screened to obtain engineered endolysins. The novel chimeric endolysin, Lys109 was selected and characterized for its staphylolytic activity. Lys109 exhibited greater bacterial cell lytic activity than its parental endolysins against staphylococcal planktonic cells and biofilms, showing highly improved activity in eliminating *S. aureus* from milk and on the surface of stainless steel. These results demonstrate that a novel chimeric endolysin with higher activity and solubility can be developed by random domain swapping and that this chimeric endolysin has a great potential as an antimicrobial agent.

Keywords: *Staphylococcus aureus*, endolysin, domain swapping, screening, antimicrobial agent

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INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium that threatens human and animal health, causing staphylococcal food poisoning and a wide range of infectious diseases, including skin infections, pneumonia, meningitis, endocarditis, and osteomyelitis (Lowy, 1998; De Lencastre et al., 2007). In particular, the global spread of methicillin-resistant *S. aureus* (MRSA) has raised serious concerns because MRSA can easily become resistant to multiple antibiotics, limiting treatment options (Chambers and Deleo, 2009). Moreover, the strong biofilm-forming ability of *S. aureus* has aggravated problems in the food and medical industries (Lewis, 2001; Otto, 2012). For these reasons, there is an urgent need to create new antimicrobials to combat *S. aureus* (Foster, 2004).

Endolysins are bacteriophage-encoded peptidoglycan hydrolases produced by bacteriophages at the end of their replication cycle to breakdown peptidoglycans of the bacterial cell wall, resulting in the release of viral progeny (Schmelcher et al., 2012a). Endolysins have been suggested as promising

antibacterial agents because purified endolysin proteins can rapidly lyse and induce death in Gram-positive bacteria when applied exogenously. Compared to classical antibiotics, endolysins have several advantages because they have narrow host specificity, high sensitivity, and a low probability to develop bacterial resistance (Borysowski et al., 2006). Gram-positive endolysins have a modular architecture with at least two separate functional regions. Generally, the N-terminal domain carries more than one catalytic domain and is attached to the C-terminal cell wall-binding domain (CBD) by a short linker (Fischetti, 2008; Schmelcher et al., 2012a). The catalytic domain determines the enzymatic activity of the endolysin, whereas the CBD positions the catalytic domain to the peptidoglycan of the target bacteria for efficient lysis by the endolysin (Loessner, 2005; Schmelcher et al., 2011).

Most endolysins of staphylococcal phages have three distinct domains: an N-terminal cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP) domain, a central N-acetylmuramoyl-l-alanine amidase (Ami_2 or Ami_3) domain, and a C-terminal SH3b domain as a CBD (Chang and Ryu, 2017). The efficacy of *S. aureus* phage endolysins killing *S. aureus* and controlling staphylococcal infection in animal models has been reported in several studies (Kerr et al., 2001; Rashel et al., 2007; Fenton et al., 2010; Gu et al., 2011). Although several *S. aureus* phage endolysins have presented promising results, poor expression levels and/or insolubility of the expressed proteins have limited the development of highly active staphylococcus-specific phage endolysins (Daniel et al., 2010). In addition, identifying a novel endolysin from *S. aureus* phages is relatively difficult because most *S. aureus*-targeting endolysins have similar domain compositions and display high amino acid sequence identity (Becker et al., 2009b; Oliveira et al., 2013; Chang and Ryu, 2017). To circumvent these problems, a number of research groups have designed truncated or chimeric versions of lysins (Manoharadas et al., 2009; Idelevich et al., 2011; Fernandes et al., 2012), but these trial-and-error strategies are time-consuming and labor-intensive in the search to find a novel endolysin with the desired properties. The modular structure of the functional domains of Gram-positive endolysin allows us to engineer endolysins through domain swapping to generate chimeric endolysins with superior properties (Diaz et al., 1990; Schmelcher et al., 2010). Endolysin engineering such as rationally designed domain recombination or random domain swapping has been endeavored. For example, Yang et al. reported an improved screening of a random domain recombination library of endolysins using controlled lysis of *E. coli* (Yang et al., 2015).

In this study, we developed an induced lysis-based screening to improve the screening efficiency further, enabling us to identify nineteen new chimeras containing different combinations of catalytic and cell wall binding domains from four *S. aureus* phage endolysins. Among them, a novel chimeric endolysin, Lys109, which showed enhanced lytic activity against *S. aureus* and other multiple staphylococcal species, was selected and characterized. This proof-of-concept study confirms the potential of the random domain swapping method to develop a novel therapeutic agent to control *S. aureus*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in **Table 1**. Staphylococcal strains were grown in tryptic soy broth (TSB) (Difco, Detroit, MI, United States) at 37°C under aerobic conditions. Baird-Parker agar plates with egg yolk tellurite (BPA; Difco) were used for the selective enumeration of *S. aureus*. *Bacillus cereus, B. subtilis, Listeria monocytogenes*, and *Streptococcus thermophilus* was cultivated in brain heart infusion (BHI) medium (Difco). Luria-Bertani (LB) medium (Difco) was used for the growth of Gram-negative strains. *Escherichia coli* DH5α and BL21 (DE3) star strains were used to clone and express proteins, respectively.

Library Construction for Random Domain Swapping

The gene encoding SPN1S lysRz (SPN1S_0028 and SPN1S_0029) was amplified from *Salmonella* Typhimurium phage SPN1S (GenBank accession number NC_016761) using the oligonucleotides listed in **Supplementary Table S1**. The gene fragment was digested with *Eco*RI-*Sal*I and inserted into the pBAD33 vector (Lim et al., 2012). To confirm the cell lysis efficiency, the pET28a_EGFP vector (Kong and Ryu, 2015) was cotransformed into competent *E. coli* BL21 (DE3) with pBAD33_SPN1S lysRz. The fluorescence of the released EGFP was measured by using a SpectraMax i3 multimode microplate reader (Molecular Devices, Sunnyvale, CA, United States) with excitation at 485 nm and emission at 535 nm. Four different *S. aureus* phage endolysins, including LysSA12, LysSA97, LysSA11, and LysSAP4, were used to construct two types of libraries (Chang and Ryu, 2017; Chang et al., 2017a). For the first library, we tried to generate chimeric endolysins containing two catalytic domains. The genes encoding four CHAP domains and those encoding three amidase domains from the four endolysins were amplified and digested with *Bam*HI/*Xho*I for insertion as the N-terminal domain of the chimeric endolysins. For the central domain of chimeric endolysins, the seven genes were amplified and digested with *Xho*I/*Bam*HI. All plasmids and primers used in this study are listed in **Supplementary Table S1**. For the second library, all genes encoding CHAPs and amidase domains were amplified and digested with only *Bam*HI to ensure diversity in the number of catalytic domains to be inserted into the endolysins. All gene fragments were randomly ligated into the *Bam*HI sites of pET28a vectors (Novagen, Madison, WI, United States) containing one of the genes encoding four different cell wall binding domains from the four endolysins. The vector libraries were cotransformed into competent *E. coli* BL21 (DE3) harboring pBAD33_SPN1S lysRz.

Screening of the Chimeric Endolysins by the Plate Lysis Method

The resulting clones from the random library were screened as described in a previous study with some modifications (Yang et al., 2015). In brief, the clones were picked and grown in 96-well plates with fresh LB broth containing 0.01 mM Isopropyl

TABLE 1 | Antimicrobial spectrum of LysSA12 and Lys109.

Species	Strains	Antimicrobial activity [a]			
		LysSA12 (pmol)		Lys109 (pmol)	
		167	16.7	167	16.7
S. aureus	Human isolate 117	+	–	++	+
	Human isolate 119	+	–	++	–
	Plant isolate 37	+	–	++	–
	Plant isolate 8	+	–	++	–
	Plant isolate 13	+	–	+	–
	Animal isolate 154	+	–	++	–
	Animal isolate 134	+	–	++	–
	Animal isolate 100	+	–	++	–
	Animal isolate 99	+	–	++	–
	Clinical isolate 1163	+	–	++	–
	Clinical isolate FMB1	+	–	++	–
	Clinical isolate FMB2	+	–	++	–
	Clinical isolate FMB3	+	–	++	–
	Mastitis cow milk isolate FMB4	++	–	++	+
	ATCC 6538	+	–	++	–
	RN4220	+	–	++	–
	ATCC 23235	+	–	++	+
	ATCC 13301	+	–	++	–
	CCARM 3090	+	–	++	–
S. hominis	ATCC 37844	+	–	++	+
S. saprophyticus	ATCC 15305	+	–	++	+
S. haemolyticus	ATCC 29970	+	–	++	–
S. capitis	ATCC 35661	+	–	++	–
S. warneri	ATCC 10209	+	–	+	–
S. xylosus	ATCC29971	+	–	+	–
S. epidermidis	CCARM 3787	++	+	++	++
Bacillus cereus	KCCM 40133	–	–	–	–
B. subtilis	168	–	–	–	–
Streptococcus thermophilus	ATCC 19258	–	–	–	–
Listeria monocytogenes	ATCC 19114	–	–	–	–
Salmonella Typhimurium	LT2	–	–	–	–
Pseudomonas aeruginosa	ATCC 27853	–	–	–	–
Cronobacter sakazakii	ATCC 29544	–	–	–	–
Escherichia coli	MG1655	–	–	–	–

[a]; ++ *clear lysis zone,* + *turbid lysis zone,* – *no lysis zone.*

β-D-1-thiogalactopyranoside (IPTG) and grown overnight at 37°C to initiate the expression of the chimeric endolysins. Afterward, 0.2% arabinose was added to the wells to express SPN1S lysRz. These cultures were dotted onto agar plates overlaid with autoclaved *S. aureus* RN 4220 and incubated for 12 h at 37°C. The clones exhibiting a clear lysis zone against *S. aureus* were screened and picked for sequencing analysis to identify the cloned chimeric endolysin.

Expression and Purification of the Endolysins

Escherichia coli BL21 (DE3) harboring chimeric endolysins were cultivated at 37°C to an optical density at 600 nm (OD_{600}) of 0.7 and the protein expression was induced by addition of 0.5 mM IPTG for 20 h at 18°C. Bacterial cells were suspended in lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride and 30% glycerol; pH 8.0) and disrupted by sonication at a duty cycle of 25% and output control of 5 (Branson Ultrasonics, Danbury, CT, United States). After centrifugation (20,000 × *g*, 30 min), the supernatant was passed through a Ni-NTA superflow column (Qiagen GmbH, Hilden, Germany), and purification of the recombinant proteins was performed according to the manufacturer's instructions. The purified protein was stored at −80°C until use after the buffer was changed to storage buffer (50 mM sodium phosphate, 300 mM NaCl and 30% glycerol; pH 8.0) using PD Miditrap G-25 (GE Healthcare, Amersham, Bucks, United Kingdom).

Lytic Activity Assay

The lytic activity of the chimeric endolysins and their original endolysins was assessed with a turbidity reduction assay (Son et al., 2012). Bacterial cells grown to the exponential phase were resuspended in reaction buffer (50 mM Tris–HCl, pH 6.5). Then, the purified proteins were added to the cell suspension at a final concentration of 300 nM, and the OD reduction of the cells was measured over time by using a SpectraMax i3 multimode microplate reader at 600 nm. The relative lytic activity was calculated after 60 min as follows: [ΔOD_{600} test (endolysin added) - ΔOD_{600} control (buffer only)]/initial OD_{600}. The antimicrobial spectrum was tested by a plate lysis assay as previously described (Chang et al., 2017a). In brief, 10 μL of diluted endolysin (167, 16.7, and 1.67 pmol) was spotted onto a freshly prepared bacterial lawn on TSA agar plates. Spotted plates were air-dried in a laminar flow hood for 15 min and incubated overnight at 37°C. The MIC of the endolysins was determined by serial dilution of the endolysins by 1:2 in 96-well plates as described previously (Andrews, 2001; Swift et al., 2015). Exponentially growing *S. aureus* CCARM 3090 was added to each well at a final concentration of 10^5 CFU/well, and the plate was incubated at 37°C for 20 h. The MIC was defined as the lowest concentration of endolysin that produced inhibition of visible growth.

Biofilm Reduction Assay

The biofilm reduction assay was performed as previously described with some modifications (Wu et al., 2003). Staphylococcal strains incubated in TSB medium supplemented with 0.25% D-glucose (Sigma-Aldrich, St. Louis, MO, United States) were prepared and subcultured in the same media in a 96-well polystyrene microplate. After incubating the microplate for 24 h at 37°C, all wells were washed with PBS. Once the biofilm formed, the experimental group wells were filled with endolysins. After incubation for 2 h at 37°C, each well was washed once with PBS and stained with 1.0% crystal violet. Next, each well was washed three times with PBS, followed by solubilization with 33% acetic acid. The absorbance of the obtained solution was measured at 570 nm, and the sessile biomass was presented as an A_{570} value.

Effect of pH and Temperature on Endolysin Activity

For the temperature stability assay of Lys109, the lytic activity was measured in reaction buffer at 25°C for 60 min after the enzyme was incubated at various temperatures (4–65°C) for 30 min. To study the effects of temperature on Lys109 enzymatic activity, 300 nM Lys109 was added into the target cell suspension, and the mixture was incubated at different temperatures (4–65°C) for 60 min. Then, the lytic activity was measured in reaction buffer at 25°C. To test the effects of pH on the activity of Lys109, 300 nM Lys109 was added to *S. aureus* CCARM 3090 cells suspended in the following buffers: 50 mM sodium acetate (pH 4.5 and 5.4), 50 mM Tris–HCl (pH 6.5–8.0), 50 mM glycine (pH 9.0), and 50 mM N-cyclohexyl-3-aminopropanesulfonic acid (pH 10.0).

EGFP Fusion Protein Binding Assay

The binding ability of EGFP_LysSA12 amidase plus CBD and EGFP_LysSA97 amidase plus CBD to *S. aureus* cells was evaluated as previously described (Loessner et al., 2002). Bacterial cells grown to the early exponential phase were harvested and resuspended in PBS. EGFP fusion proteins were incubated with the cells for 5 min at 25°C. The mixture was washed twice with PBS to remove unbound protein and was moved to a 96-well plate to measure the fluorescence using a SpectraMax i3 multimode microplate reader (excitation at 485 nm and emission at 535 nm). The OD_{600} of the cells was measured to normalize the fluorescence by calculating the whole-cell fluorescence per OD_{600}.

Antimicrobial Activity Assay in Food Samples

The lytic activity of Lys109 against the MRSA CCARM 3090 strain was tested in commercial whole-fat pasteurized milk as previously described (Chang et al., 2017a). A milk sample was inoculated with exponentially growing MRSA CCARM 3090 cells (approximately 10^5 CFU/mL). Before the addition of Lys109 and LysSA12 at 0, 30, 300, 900, and 1500 nM, the milk samples were preincubated with the bacteria at 25°C for 1 h to allow the bacteria to adapt to the milk. Each milk sample was then incubated at 25°C for an additional hour. Viable bacterial cells (CFU/mL) were counted every 15 min after the addition of Lys109 and LysSA12 by plating each sample on a BPA plate and incubation at 37°C for 24 h.

Antimicrobial Activity Assay on Stainless Steel

The lytic activity of Lys109 against the MRSA CCARM 3090 strain was tested on stainless steel as previously described (Cha et al., 2019). A stainless steel coupon with a size of 2 × 2 cm^3 was sterilized in an autoclave. Exponentially growing MRSA CCARM 3090 bacterial cells were harvested and resuspended in PBS to a final concentration of approximately 10^5 CFU/mL. Prepared bacterial cells were pipetted onto the stainless steel surface and dried for 1 h on a clean bench. Subsequently, the stainless steel samples were treated with Lys109 (0–100 nM) and left for 60 min at 25°C. For the negative control, PBS was used instead of Lys109 solution. *S. aureus* cells were detached from the surface by agitation in PBST for 2 min with a bench-top vortex mixer at maximum speed. Cell suspensions were serially diluted and plated onto BPA plates and incubated at 37°C for 24 h.

Statistical Analysis

GraphPad Prism (version 5.01) was used to conduct statistical analysis. One-way analysis of variance (ANOVA) followed by one-way Tukey's test for all pairwise comparisons (95% confidence interval) was performed. The data are presented as the means with standard deviations. A P-value < 0.05 was considered statistically significant.

RESULTS

Development of a Random Domain Swapping Method

The overall scheme of random domain swapping method was presented in **Figure 1A**. The protocol was established based on a 96 well plate format for the rapid and efficient screening, taking advantage of a *S.* Typhimurium phage SPN1S lysis cassette composed of an endolysin and Rz/Rz1-like proteins (SPN1S lysRz) (Lim et al., 2012). SPN1S lysRz has been reported to cause host cell lysis and release viral progeny at the end of phage life cycle. To evaluate the cell lysis efficiency of SPN1S lysRz, the amount of EGFP protein released from lysed *E. coli* was measured. Fluorescence significantly increased after induction with arabinose (**Figure 1B**), indicating that SPN1S lysRz can cause the rapid lysis of *E. coli* cells from within, thereby releasing accumulated proteins in the cytosol. To identify an active chimeric endolysin from a large random library, the lytic efficacy of the released proteins was evaluated by their ability to form a clear zone on an agar plate overlaid with heat-killed *S. aureus* cells. As positive controls, four different *S. aureus* phage endolysins (LysSA11, LysSA97, LysSAP4, and LysSA12) were expressed in the presence of pBAD33_SPN1S lysRz. The clear zones were visualized on the agar plate depending on their activities, whereas clones without pBAD33_SPN1S lysRz did not show a clear zone (**Figure 1C**). These results suggest that SPN1S lysRz-induced lysis of *E. coli* allowed active chimeric endolysins to form clear zone on agar plates containing target bacteria and that this system can be used as a method for the successful screening of novel chimeric endolysins.

Isolation of a Novel Endolysin Lys109

The random library containing 480 clones were cotransformed into *E. coli* containing the SPN1S lysRz-harboring vector and

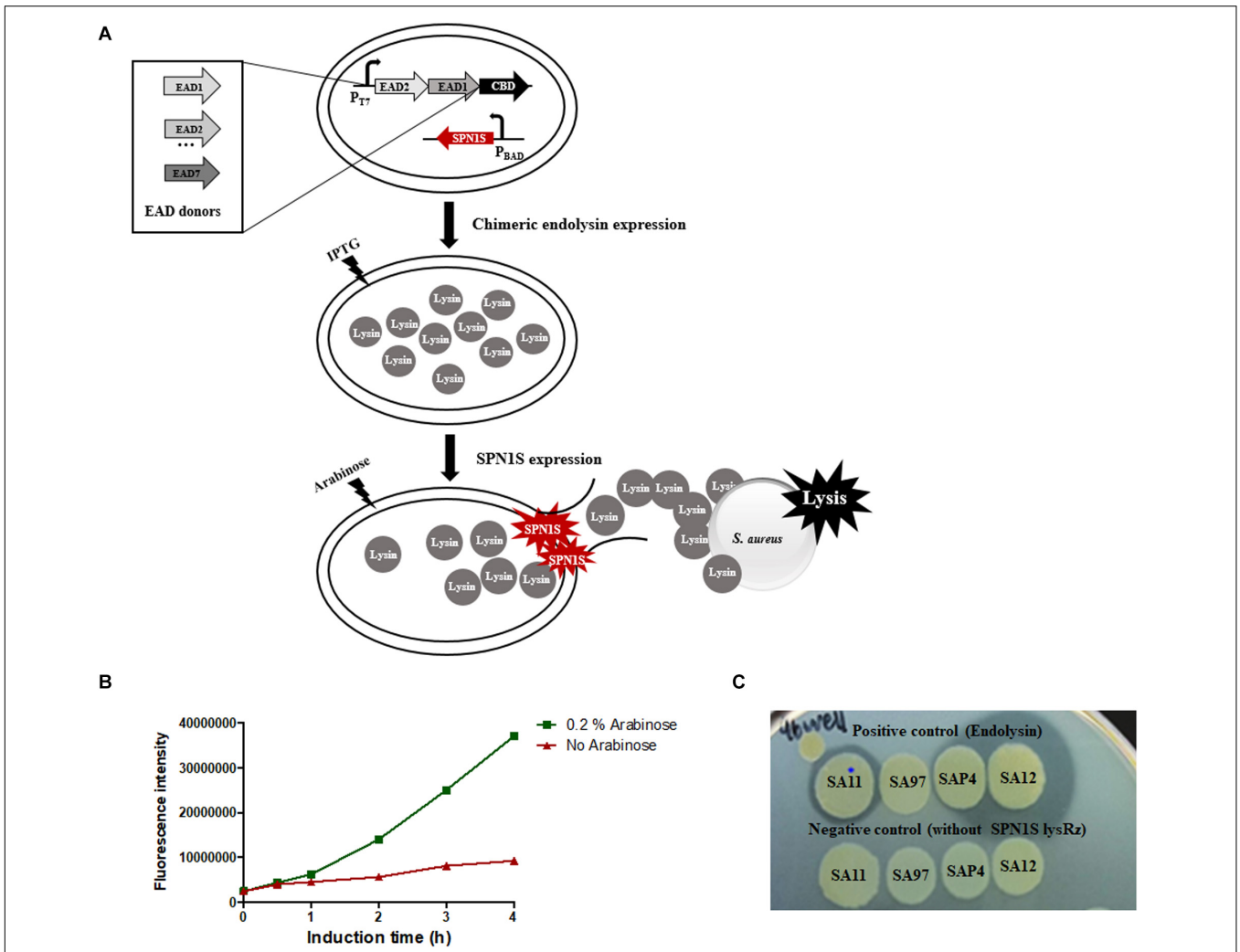

FIGURE 1 | Development of screening system on a 96-well microplate. **(A)** Scheme of the random domain screening method. **(B)** EGFP released from SPN1S-induced lysis in host *E. coli* cells. Cells without induction IPTG for EGFP expression and arabinose for SPN1S lysRz expression) were used as a control. **(C)** Clones for positive and negative controls were cultured with 0.2% arabinose overnight on the lawn of autoclaved *S. aureus* RN4220.

applied to an agar plate containing *S. aureus* for screening. The clones displaying clear lysis zones were sequenced to determine the combination of the EADs (**Supplementary Table S2**). Most of the selected clones contained a CHAP domain from LysSA12 or LysSA11, which showed high staphylolytic activity (Chang et al., 2017a,b). In particular, LysSA12 CHAP domain-containing clones, which account for 68% of selected clones, showed a large and clear lysis zone. There was also a single clone containing a LysSAP4 CHAP domain. These results suggest that CHAP domain is necessary to degrade *S. aureus* cell wall peptidoglycan, and this is consistently observed in other chimeric endolysins to control *S. aureus* (Daniel et al., 2010; Schmelcher et al., 2012c; Yang et al., 2014). Among LysSA12 CHAP domain-containing clones, five promising chimeric endolysins were

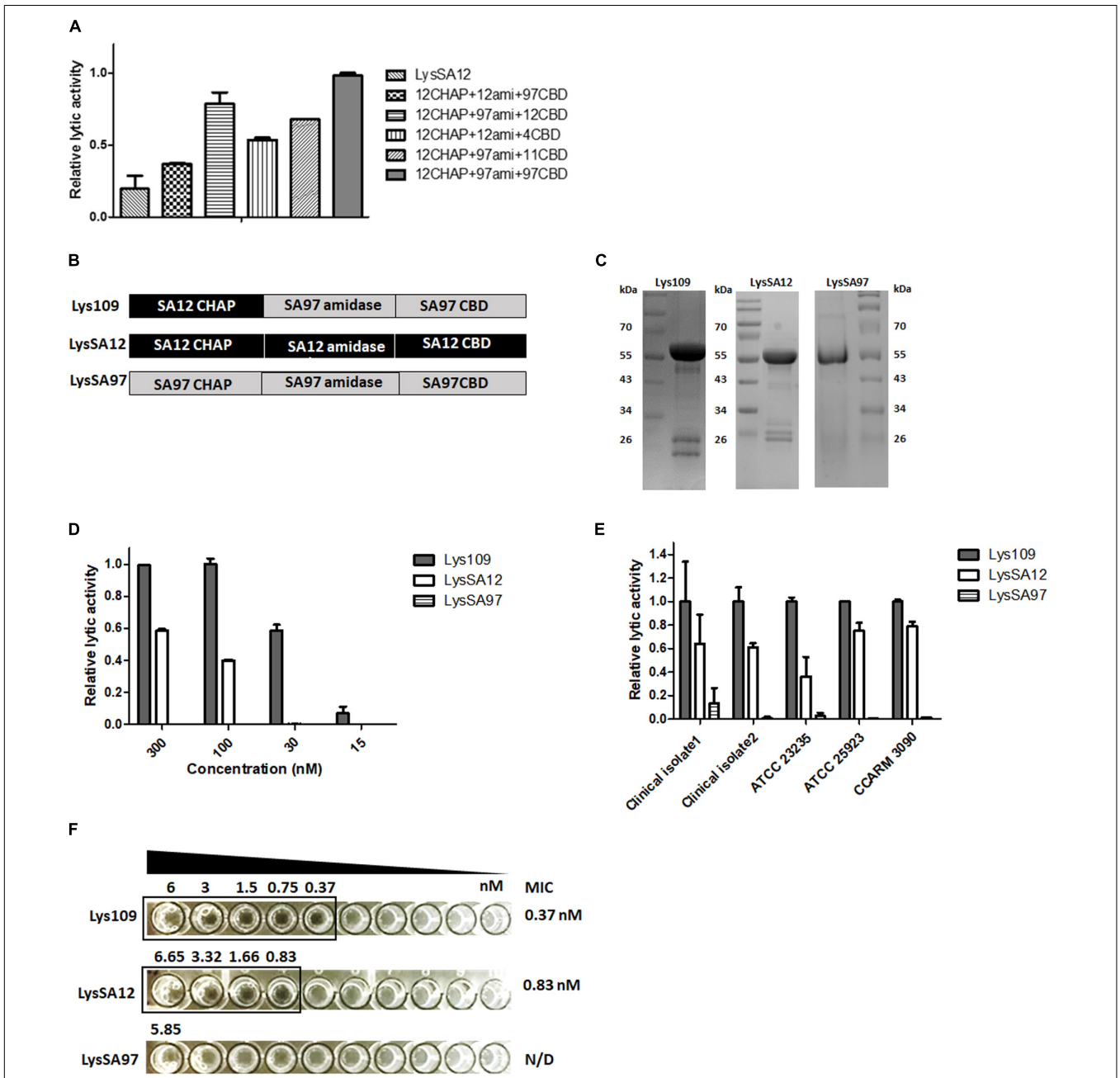

FIGURE 2 | Lytic activity comparison of Lys109 with parental endolysins. **(A)** Comparison of the relative lytic activity of four other chimeric endolysins and LysSA12 with Lys109 (300 nM each) against *S. aureus* CCARM 3090. **(B)** Schematic representation of Lys109, LysSA12 and LysSA97. **(C)** SDS-PAGE analysis of Lys109, LysSA12, and LysSA97. **(D)** Relative lytic activity of Lys109, LysSA12 and LysSA97 against *S. aureus* CCARM 3090 at different concentrations. **(E)** Relative lytic activity of Lys109, LysSA12 and LysSA97 (300 nM each) against various *S. aureus* strains. **(F)** MIC values of Lys109, LysSA12 and LysSA97 against *S. aureus* CCARM 3090.

selected for the further comparative analysis (**Figure 2A**). All selected proteins were expressed in *E. coli* in soluble form and evaluated for their lytic activity against *S. aureus*. As a result, a chimeric endolysin consisting of a LysSA12 CHAP domain in the N-terminal region, a LysSA97 amidase domain at the central and a LysSA97 CBD in the C-terminal region showed the highest lytic activity among the five candidates, and was designated Lys109 (**Figure 2B**). BLAST analysis revealed that Lys109 has 78% overall amino acid sequence identity with an amidase from *S. aureus* phage StauST398-1 (Van Der Mee-Marquet et al., 2013) and 80% with endolysins from ΦB166 and ΦB236 *S. aureus* phage (Botka et al., 2015). Although the three endolysins showed high similarity with Lys109, they have not yet been studied, suggesting that further research on Lys109 will be meaningful. Moreover, LysSA12, a CHAP domain donor of Lys109, has high amino acid similarity with LysH5 of *S. aureus* phage vB_SauS_phiIPLA88 (98% identity to LysSA12) and *S. aureus* Φ11 endolysin (96% identity to LysSA12) (Sass and Bierbaum, 2007; García et al., 2010). These endolysins have a conserved catalytic triad (C32, H95, and Q112) in their CHAP domains (**Supplementary Figure S1**). Considering that the lytic activity of *S. aureus* endolysin depends mostly on a CHAP domain, we selected Lys109 to investigate the effect of domain replacement on the activity of the endolysin.

Lytic Activity of Lys109 in Comparison With Its Parental Endolysins

Lys109 and its parental endolysins, LysSA12 and LysSA97, were highly expressed as a soluble form in *E. coli* and were purified via Ni-NTA affinity chromatography. The predicted molecular weights of Lys109, LysSA12 and LysSA97 were approximately 54 kDa and the proteins migrated as expected in SDS-PAGE gel (**Figure 2C**). The antibacterial activity of Lys109 was compared with those of LysSA12 and LysSA97 at various concentrations (**Figure 2D**). Lys109 exhibited clear cell lysis against *S. aureus* CCARM 3090, displaying at least 2-fold higher lytic activity than that of LysSA12 and LysSA97 at all tested concentrations. LysSA12 did not show lytic activity at concentrations below 30 nM and LysSA97 barely exhibited staphylolytic activity at all tested concentrations. These results indicate the superiority of lytic activity of LysSA12 CHAP over LysSA97 CHAP despite their sequence similarity (44% identity). Comparative analysis of the lytic activity of Lys109 with its parental endolysins against other *S. aureus* strains including clinical isolates and MRSA also showed an evident improvement in the lytic activity of Lys109 (**Figure 2E**). The MIC of Lys109 was compared with those of its parental endolysins. LysSA97, a donor for the amidase domain and CBD of Lys109, did not show inhibition of cell growth at the maximum concentrations available (5.85 μM) (**Figure 2F**), leading us to exclude LysSA97 in the following experiments. LysSA12 inhibited the growth of *S. aureus* CCARM 3090 at concentration of 0.843 μM. The MIC of Lys109 was 0.375 μM, which was at least 2.25-fold lower than that of LysSA12, indicating that the antimicrobial activity of Lys109 was significantly improved compared to its parental endolysins.

Biofilm Reduction Activity of Lys109

The biofilm reduction efficacy of Lys109 against biofilms formed by *S. aureus* CCARM 3090 and *S. aureus* RN4220 was evaluated by a crystal violet staining-based assay (**Figure 3**). Lys109 showed biofilm reduction activity in a dose-dependent manner, and Lys109 appeared to have a higher biofilm reduction efficacy than LysSA12. When 300 nM of endolysins were added to the biofilms, Lys109 exhibited more than 3-fold enhanced efficacy in removing the biofilms compared to LysSA12. These results demonstrate that Lys109 has strong lytic activity against not only planktonic cells but also biofilms, which is an important contributing factor for many treatment failures (Otto, 2013).

Temperature and pH Effects on the Enzymatic Activity of Lys109

The thermostability of endolysins was determined (**Supplementary Figure S2**). Lys109 retained over 95% of its activity after 1 h of incubation at 4 to 37°C, and the lytic activity of Lys109 started to decrease at 45°C. Approximately 40% decrease in its hydrolytic activity was observed at 50°C, and higher temperatures (55 and 65°C) caused complete inactivation of Lys109. LysSA12 showed similar pattern of thermal stability to that of Lys109, indicating that the structural stabilization by the fusion of LysSA12 CHAP to LysSA97 amidase domain plus CBD might not be the reason of the enzymatic improvement of Lys109. Next, the effects of temperature and pH on the lytic activity of Lys109 were evaluated to determine the optimum working conditions of Lys109 (**Figures 4A,B**). The maximal activity of Lys109 was exhibited at 25–37°C and pH 6.5–9.0. The wide optimum pH range of Lys109 suggest that Lys109 can be used for a wide variety of applications, including foods associated with a high risk for *S. aureus* contamination, such as milk products (Marino et al., 2000) and disinfectants for hospital cleaning (Dancer, 2008).

Antibacterial Spectrum of Lys109

The antimicrobial activity of Lys109 was examined against staphylococcal strains other Gram-positive and Gram-negative bacteria with different amounts of endolysins, and was compared to that of LysSA12 (**Table 1**). Lys109 displayed effective lytic activity against all tested staphylococcal strains but not the other Gram-positive and Gram-negative bacteria tested. Although Lys109 has the same antibacterial spectrum as LysSA12, Lys109 showed higher lytic activity against most staphylococcal strains tested compared with LysSA12. These results demonstrated that Lys109 retains the specificity of the original endolysin but has stronger antimicrobial properties.

Efficacy of Lys109 Against *S. aureus* in Milk

The comparative antibacterial activity of Lys109 and LysSA12 against a MRSA (*S. aureus* CCARM 3090) strain was examined at various concentrations in milk. Milk was chosen for the test because they have been frequently implicated in staphylococcal foodborne illnesses (Kadariya et al., 2014). In milk artificially contaminated with *S. aureus*, treatment with LysSA12 did not

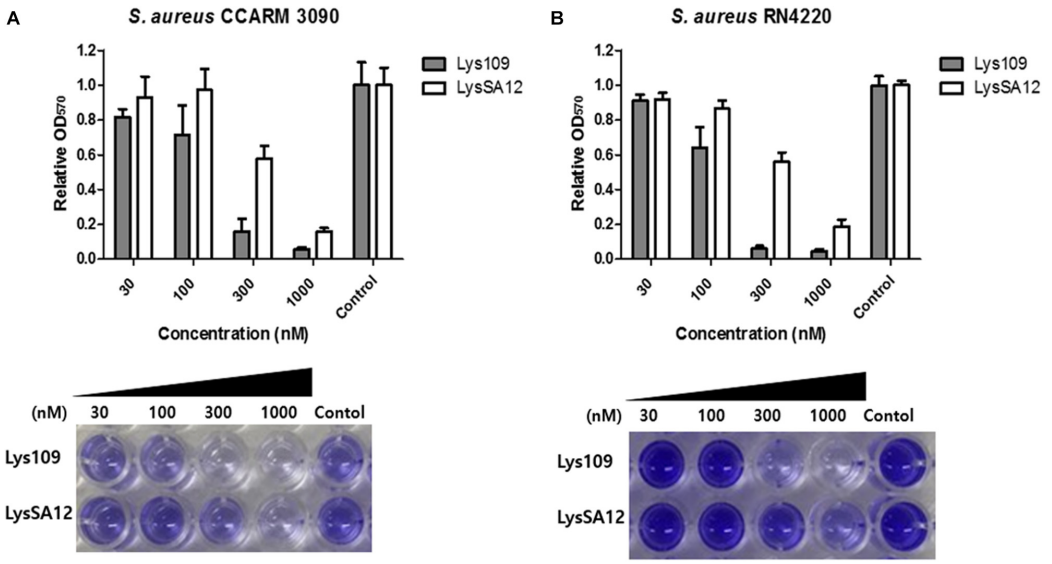

FIGURE 3 | Biofilm reduction activity of Lys109 and LysSA12. Biofilms formed by **(A)** *S. aureus* CCARM 3090 and **(B)** *S. aureus* RN4220 were treated with various concentrations of Lys109 and LysSA12 and visualized by staining biofilms with crystal violet. Dark staining indicates the biofilm that was maintained after treatment with the endolysin. Light or no staining indicates successful removal of the biofilm. Control indicates the sample treated with buffer without endolysins.

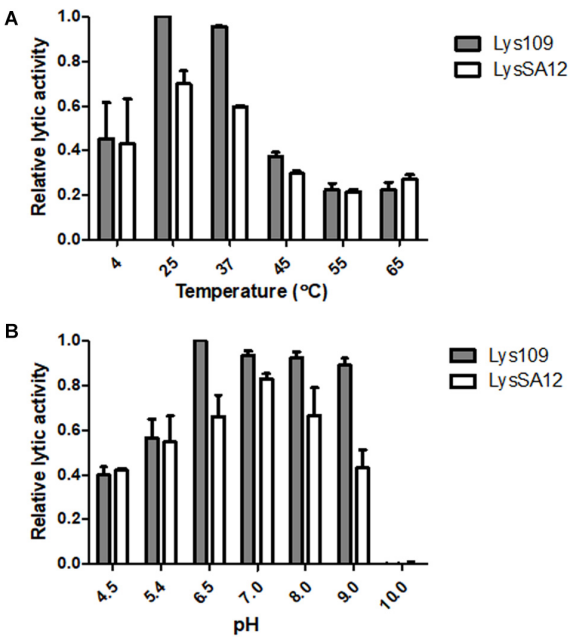

FIGURE 4 | The effects of temperature and pH on the lytic activity of Lys109 and LysSA12. The optimum **(A)** temperature and **(B)** pH of Lys109 and LysSA12 were examined by incubation of the enzymes (300 nM each) with target cells at different temperatures and in different pH buffers, respectively. The relative lytic activities were calculated using the activity of Lys109 assayed in a buffer at pH 6.5 and 25°C, which showed maximal activity.

show any reduction in CFU at all tested concentrations even though LysSA12 exerted lytic activity at 100 nM in reaction buffer (**Figure 5A**), demonstrating the importance of *in vivo* experiments to evaluate the potential of a new antimicrobial. Similarly, LysH5, a LysSA12 homolog, could not kill bacteria cells in milk at 0.15 μM, and only 1-log reduction was observed with 0.8 μM LysH5 (Obeso et al., 2008; García et al., 2010). On the other hand, treatment with 300 nM of Lys109 showed an apparent inhibitory effect on *S. aureus* within 15 min and resulted in a 2-log reduction of bacterial cells after 1h (**Figures 5B,C**). In addition, the number of *S. aureus* in milk decreased below the detection limit with 900 nM Lys109 within 45 min (**Figure 5B**), indicating improved antimicrobial activity of the chimeric endolysin compared to parental endolysins. These findings showed that Lys109 has high staphylolytic activity in a complex biomatrix as well as in buffer and suggest that Lys109 has great potential as an antimicrobial agent to control *S. aureus* from dairy products.

Efficacy of Lys109 Against *S. aureus* on Stainless Steel

Staphylococcus aureus is a common bacterium encountered in hospital-acquired and device-associated infections (Gwisai et al., 2017). Stainless steel is commonly used for medical devices, and previous reports revealed that MRSA can be viable and grow on it, leading to human infections (Michels et al., 2015; Craft et al., 2019). In this regard, the staphylolytic efficacy of Lys109 was evaluated and compared with its parental endolysin, LysSA12, on stainless steel coupons artificially contaminated with *S. aureus* CCARM 3090. At 100 nM, treatment with Lys109 on the surface of a stainless steel coupon caused the number of bacterial cells to decrease below the detection limit, whereas its parental endolysin was less effective in bacterial cell killing, resulting in only a 1-log bacterial reduction after 1 h (**Figure 6**). These results suggest the possible use of Lys109 as a disinfectant in hospital settings.

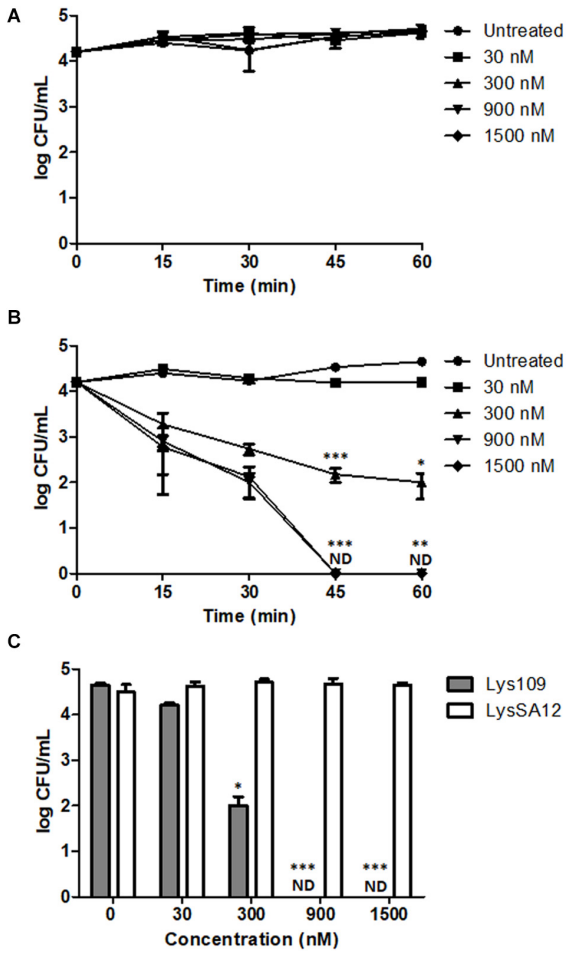

FIGURE 5 | Antibacterial activity of Lys109 and LysSA12 against *S. aureus* CCARM 3090 in milk. **(A)** LysSA12 and **(B)** Lys109 were added to milk artificially contaminated with *S. aureus* CCARM 3090 at different concentrations. Bacterial cells were counted every 15 min for 1 h. **(C)** The number of *S. aureus* CCARM 3090 cells in milk was counted after 1 h of treatment with Lys109 and LysSA12 at concentrations of 0 nM (negative control), 30, 300, 900, and 1500 nM. ND, not detected. Asterisks indicate significant differences (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

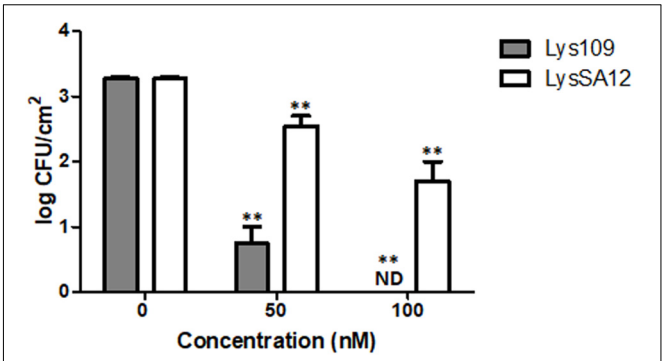

FIGURE 6 | Antibacterial activity of Lys109 and LysSA12 against *S. aureus* CCARM 3090 on stainless steel. The number of *S. aureus* CCARM 3090 cells was counted after treatment with Lys109 and LysSA12 at concentrations of 0 nM (negative control), 50 and 100 nM. ND, not detected. Asterisks indicate significant differences (**$P < 0.01$).

DISCUSSION

The emergence of multidrug-resistant *S. aureus* has called for novel therapeutic options beyond conventional antibiotics. Endolysins from staphylococcal phages have been proposed as promising alternatives to combat *S. aureus* due to their near species specificity and low probability of developing bacterial resistance (Kashani et al., 2018). In this regard, many researchers have tried to isolate *S. aureus* phages and to identify novel endolysins within the genomes of isolated phages. However, this approach is time-consuming and usually inefficient because staphylococcal endolysins, which are generally classified into five major groups according to their domain architectures (Chang and Ryu, 2017), have more than 90% amino acid sequence identity within each group (Becker et al., 2009b). Besides, poor expression and low solubility levels have further aggravated the situation of getting highly active *S. aureus* endolysins (Manoharadas et al., 2009; Daniel et al., 2010; García et al., 2010). For these reasons, developing a novel *S. aureus*-targeting endolysin with desired properties is of both commercial and academic interest. In this study, we propose an endolysin engineering strategy based on the random domain swapping method to get highly active *S. aureus*-targeting endolysins.

Our screening method involves several noteworthy features as follows. First, we utilized the lytic ability of *S.* Typhimurium phage SPN1S lysRz cassette to lyse *E. coli* cells from within to release expressed endolysins from the cytosol. The SPN1S lysRz cassette consists of an endolysin and two component spanins (Rz and Rz1) which are reported to be essential for disrupting the outer membrane in this final step of host lysis (Rajaure et al., 2015). The co-expression of SPN1S lysRz resulted in strong cell lysis and we were rapidly able to screen highly active chimeric endolysins using 96-well plate format. Second, we constructed two different types of random domain libraries to increase the library diversity, one with variable number of EADs in a random orientation, and the other with only two EADs. A total 19 clones were selected from the libraries based on their strong lytic profiles on *S. aureus* lawns. Sequence analysis revealed that all 19 clones have one or two CHAP domains at the N-terminus and 13 out of the 19 selected clones harbored an amidase domain in the middle of the chimeric endolysins. Consistent with previous reports (Becker et al., 2009a; Schmelcher et al., 2012b; Son et al., 2018), our results indicate that the N-terminal CHAP domain is essential for lysis of *S. aureus* cells and that the amidase domain at the central region may enhance the overall lytic activity of endolysins. Third, the use of heat-killed *S. aureus* cells allowed us to distinguish clearly active chimeric endolysins among clones in the screening process. When *E. coli* and *S. aureus* were co-cultured, it gave rise to false positive clones, some of which showed clear zone on the lawn of *S. aureus* but produced insoluble or inactive form of proteins. Considering

that *E. coli* grows faster than *S. aureus* in mixed culture (Fujikawa and Sakha, 2014), we speculate that *E. coli* might inhibit the growth of *S. aureus*, forming inhibition-like zone on the *S. aureus* lawn.

We found that this screening strategy was successful for developing a novel chimeric endolysin and Lys109, which showed the most effective staphylolytic activity, was selected from the random libraries. Lys109 was composed of a LysSA12 CHAP domain, a LysSA97 amidase domain, and a LysSA97 CBD. The efficacy of Lys109 to remove staphylococcal planktonic cells and biofilms was much stronger than that of its parental endolysins, LysSA12 and LysSA97. Then, how Lys109 has superior lytic activity to its parental endolysins? One possible reason is that the increased binding ability of the chimeric endolysin might have led to the improvement of antibacterial activity (Son et al., 2018). Indeed, LysSA97 amidase plus CBD displayed higher binding affinity to the target bacteria than LysSA12 amidase plus CBD (**Supplementary Figure S3**), suggesting that LysSA97 amidase plus CBD increases the lytic activity of LysSA12 CHAP by enhancing its accessibility to the target bacteria. Alternatively, the peptidoglycan fragment generated by the initial LysSA12 CHAP digestion could be more sensitive to the LysSA97 amidase domain plus CBD than the LysSA12 amidase plus CBD (Becker et al., 2009a). Further structural and biophysical studies of Lys109 will be needed to verify the exact molecular mechanism of enhanced lytic activity provided by domain swapping.

Lys109 showed much stronger antimicrobial activity than its parental endolysin, LysSA12 in milk. The treatment with 300 nM of Lys109 for 1 h showed 2-log reduction of bacterial cells in milk and 900 nM of Lys109 was sufficient to reduce the staphylococcal cells to undetectable levels within 45 min. Several other peptidoglycan hydrolases also have been examined for their antimicrobial activity in milk, but they generally showed low lytic activity in milk (Schmelcher et al., 2015; Verbree et al., 2018). LysH5, a LysSA12 homolog, could not kill bacterial cells in milk with 0.15 μM and only a 1-log reduction was observed with 0.8 μM LysH5 (Obeso et al., 2008; García et al., 2010). The antimicrobial activity of LysSA97, a donor for an amidase and a CBD of Lys109, was also marginal when 1.88 μM of the protein was added in milk (Chang et al., 2017b). More recently, LysSA11 derived from *S. aureus* phage SA11 showed only a 1-log reduction of staphylococcal cell counts in milk when it was added at 2.25 μM for 1 h (Chang et al., 2017a). These results indicate the potential of Lys109 as antimicrobial additives for milk products. Besides, we observed that Lys109 has strong antimicrobial activity on stainless steel. The result demonstrates that stainless steel, the material for medical devices, does not significantly affect the staphylolytic activity of Lys109 and that Lys109 can be possibly used as a disinfectant in clinical settings.

In summary, we proposed an effective screening method to find a novel chimeric endolysin with higher lytic activity and solubility through a random domain swapping of *S. aureus* endolysins. Lys109 was selected from random libraries and showed much stronger lytic activity against staphylococcal strains than its parental endolysins, LysSA12 and LysSA97. Moreover, Lys109 effectively removed staphylococcal biofilms and significantly reduced the number of *S. aureus* cells in milk and on the surface of stainless steel. These results suggest that the random domain swapping method can provide an opportunity for researchers to generate a novel and potent chimeric endolysin with minimal effort. Our strategy therefore holds considerable potential for medical and biotechnological applications to combat multidrug-resistant bacteria such as *S. aureus*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

BS and SR conceived and designed the experiments. BS performed the experiments and analyzed the data. YL participated in the experiments. BS and MK wrote the manuscript. SR revised the manuscript. All authors have read and accepted the final manuscript.

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frontiers in Microbiology

ORIGINAL RESEARCH
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An Optimized Bacteriophage Cocktail Can Effectively Control *Salmonella in vitro* and in *Galleria mellonella*

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Salmonella spp. is a leading cause of gastrointestinal enteritis in humans where it is largely contracted via contaminated poultry and pork. Phages can be used to control *Salmonella* infection in the animals, which could break the cycle of infection before the products are accessible for consumption. Here, the potential of 21 myoviruses and a siphovirus to eliminate *Salmonella in vitro* and *in vivo* was examined with the aim of developing a biocontrol strategy to curtail the infection in poultry and swine. Together, the phages targeted the twenty-three poultry and ten swine prevalent *Salmonella* serotype isolates tested. Although individual phages significantly reduced bacterial growth of representative isolates within 6 h post-infection, bacterial regrowth occurred 1 h later, indicating proliferation of resistant strains. To curtail bacteriophage resistance, a novel three-phage cocktail was developed *in vitro*, and further investigated in an optimized *Galleria mellonella* larva *Salmonella* infection model colonized with representative swine, chicken and laboratory strains. For all the strains examined, *G. mellonella* larvae given phages 2 h prior to bacterial exposure (prophylactic regimen) survived and *Salmonella* was undetectable 24 h post-phage treatment and throughout the experimental time (72 h). Administering phages with bacteria (co-infection), or 2 h post-bacterial exposure (remedial regimen) also improved survival (73–100% and 15–88%, respectively), but was less effective than prophylaxis application. These pre-livestock data support the future application of this cocktail for further development to effectively treat *Salmonella* infection in poultry and pigs. Future work will focus on cocktail formulation to ensure stability and incorporation into feeds and used to treat the infection in target animals.

Keywords: *Salmonella*, gastrointestinal enteritis, *Galleria mellonella*, bacteriophage, bacteriophage therapy

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INTRODUCTION

Non-typhoidal *Salmonella* spp. are a leading cause of acute gastroenteritis in humans. Annually, *Salmonella* infection is responsible for ∼155,000 deaths and 93.8 million cases of food poisoning worldwide, of which 85% of all cases are foodborne (Majowicz et al., 2010; Eguale et al., 2015; Balasubramanian et al., 2019). The major route of transmission to humans is via the consumption

of food products contaminated with *Salmonella*, especially through poultry and pork related products (Foley et al., 2008). Chickens, turkeys and pigs can become infected with *Salmonella* from contaminated feeds, environment or through contact with other infected animals in the pen (Atterbury et al., 2007). Once the animals are infected, they can remain asymptomatic or develop enteric infection symptoms. Either way, their guts become colonized with *Salmonella* and the bacterium can spread between animals via fecal-oral route (Bonardi, 2017; Martínez-Avilés et al., 2019). In addition to transmission, there is a risk of carcass-to-carcass contamination with *Salmonella* during slaughtering and meat processing (Smith et al., 2018). Consequently, each stage of processing from farm to fork presents a potential risk point of *Salmonella* contamination and infection (Akil and Ahmad, 2019). Thus, breaking the cycle of infection within the food chain before the products are available for consumption represents a desirable approach to prevent and control this infection in humans.

Salmonella serotypes commonly associated with poultry and pig infections, and human to human infection via faecal-oral route are *S.* Typhimurium, *S.* 1,13,23:i:, *S.* Enteritidis, *S.* Infantis, *S.* Ohio, and *S.* Seftenberg (Antunes et al., 2016; EFSA, 2018; Ferrari et al., 2019). An increasing number of strains from these serotypes are becoming resistant to the front-line antibiotics used to control *Salmonella* on farms, including to the last resort antibiotic, colistin (Anjum et al., 2016). Worryingly, The European Food Safety Authority (EFSA) reported that 94.4% of 659 *S.* Infantis strains isolated from broilers were resistant to one or more antibiotics, and 64.2% of 123 *S.* Typhimurium strains isolated from pig carcasses were multi-drug resistant (MDR; EFSA, 2018). As a consequence of this, MDR strains have entered the human food chain and alternative antimicrobials are therefore needed to treat and control the spread of MDR *Salmonella* strains in both animals and humans.

Bacteriophages (phages) are natural viruses of bacteria and as such can be developed to offer a viable alternative to antibiotics (Salmond and Fineran, 2015; Czaplewski et al., 2016). Studies have shown that phages are able to lyse MDR *Salmonella* strains (Jung et al., 2017; Thanki et al., 2019; Li et al., 2020) and hence, could be a tool to limit the spread of these strains in the food chain. Due to this increased need for novel antimicrobials, research into the therapeutic use of lytic phages, known as "phage therapy," has been growing exponentially (Nobrega et al., 2015). To be used most effectively in therapy, phages can be combined as "cocktails" to broaden their host range coverage, improve killing efficiency or limit the development of phage resistance (Chan et al., 2013). Many phage cocktails have been designed against *Salmonella* and their efficacy has been tested in challenge studies both in swine and poultry (Zhang et al., 2015; Martinez et al., 2019). They have been deployed at various intervention points and a pre-slaughter study showed administering a four-phage cocktail in feed ($\sim 10^7$ PFU/g) was able to reduce *Salmonella* colonization in the caecum of chickens by 1 $\log_{10}$ CFU/g over 14 days (Sklar and Joerger, 2001). Similarly, a sixteen-phage cocktail (5×10^9 PFU) administered simultaneously with a *S.* Typhimurium (5×10^9 CFU) reduced *Salmonella* colonization by 2–3 $\log_{10}$ CFU/g in the caecum, ileum and tonsils of weaning pigs (Wall et al., 2010). In another study, a five-phage cocktail administered post slaughter effectively reduced *S.* Enteritidis on chicken skin by 1.0 log CFU/cm^2 when administered at the somewhat high multiplicity of infection (MOI) of 10,000 (Hungaro et al., 2013). Finally and rather encouragingly, a similar study showed application of a four-phage cocktail at MOIs of 10 and 100 on pig skin contaminated with *S.* Typhimurium reduced bacterial numbers to below detection levels after 96 h (Hooton et al., 2011).

Although studies have highlighted the use of phage cocktails in reducing *Salmonella* numbers in both pre- and post-slaughter settings, few phage products are available on the market to control infection in poultry and pigs (Żbikowska et al., 2020). For a product to be effective in this setting, it needs to have optimal broad host-range activity to effectively eliminate the diverse *Salmonella* serotypes in animals. Therefore, to address this paucity of information in the control of *Salmonella* infection, here, a novel three-phage cocktail was developed to carry out the first steps needed for the ultimate use of phages as a therapeutic feed additive. The cocktail contains two broad host-range myoviruses and a siphovirus, all of which target prevalent poultry and swine isolates. Clearly, activity of phages *in vitro* may vary *in vivo* and thus further testing in animals is needed. Testing in livestock is expensive and time consuming so to circumvent these difficulties and to reduce the volume of work needed in livestock, the phage cocktail was extensively tested in a *Galleria mellonella* larva *Salmonella* infection model. Previous data from our laboratory and others have shown that the *Galleria* model is useful and that it correlates to large scale animal models. More broadly, the *G. mellonella* model is cheap and is a valuable biological tool to study the virulence and pathogenicity of pathogens including *Salmonella*, and pharmacokinetics of anti-infectives including phage therapy (Thomas et al., 2013; Nale et al., 2016a, 2020). In this study, *G. mellonella* larvae were colonized with representative isolates and detail evidence of the efficacy of different phage therapeutic regimens to prevent and reduce colonization in the model was obtained and is presented.

MATERIALS AND METHODS

Bacterial Strains, and Phage Collation, Isolation and Propagation

In total, 35 *Salmonella* strains were examined in this study. This consisted of twenty-three poultry and ten swine *Salmonella* strains, which were isolated and kindly provided by the Animal and Plant Health Agency (APHA) Weybridge, United Kingdom (**Supplementary Tables S1, S2**). The phage propagating host, *Salmonella enterica* serovar Typhimurium SL1344 (accession number FQ312003) was obtained from Dr. Primrose Freestone at University of Leicester, and was previously characterized in our laboratory and elsewhere (Viegas et al., 2013; Thanki et al., 2019). *S. enterica* serovar Typhimurium T4, is a routine laboratory strain and was used as a reference strain for phage cocktail development and testing *in vivo*. Bacteria were routinely grown on Xylose Lysine Deoxycholate (XLD) agar (Oxoid, United Kingdom) for

18 h at 37°C before being cultured in Luria-Bertani (LB) broth (Oxoid, United Kingdom) for 18 h at 37°C at 100 rpm.

Twenty-two *Salmonella* phages were tested here. Twenty, were previously isolated and characterized in our laboratory while two, were also previously isolated and characterized in Thailand (Thanki et al., 2019; Phothaworn et al., 2020). All bacterial and phage strains were preserved long term in Viabank cryogenic vials (Abtek Biologicals Ltd., United Kingdom) at −80°C.

Phage Propagation

To propagate the phages, individual phages were added to separate exponentially growing liquid cultures of SL1344 at OD_{600} ∼0.2 (10^8 CFU/mL) in LB broth at MOI of 0.1, and further incubated at 37°C with shaking at 100 rpm for 6 h. Cultures of lysed bacterial cells were centrifuged at 5,000 *g* for 15 min, supernatants filtered through 0.2 μm pore size filters (Merch Millipore Ltd. Cork, Ireland) and temporarily stored at 4°C. Phage titers were determined using double agar method with a top bacterial lawn prepared in 4 ml 0.7% LB agar and 150 μL of overnight cultures (produced by inoculating one colony of the bacterial cultures in to 5 mL LB broth and incubated at 37°C for 18–24 h) cast on 1% LB agar 90 mm plates (Kropinski et al., 2009) and expressed as PFU/ml. Equal volumes of phage lysates at the same titers were mixed to form a cocktail.

Phage Host Range and Virulence Assays on Chicken and Pig Isolates

The host range of each phage was determined by adding 10 μL 10^8 PFU/mL volumes of lysates to confluently grown bacterial strains prepared as above and incubated aerobically for 18 h at 37°C. Plates were examined for bacterial lysis from three biological and technical replicates.

Five resistant clones obtained from each single phage infection were picked and purified by sub-culturing five times on fresh XLD medium. Each purified clone was confirmed to be resistant if they were no longer susceptible to infection with 10^8 PFU/mL of the wild-type phage in host range spot testing assay as described above. Confluently grown resistant clones were prepared as above and 10 μL of the wild-type phages were applied to them. Lysis zones were observed after incubation aerobically for 24 h at 37°C.

Phage virulence was determined using killing assay on cultures of SL1344. To do this, 180 μL triplicates of each bacterial culture were produced by diluting 1:10 overnight cultures in sterile LB broth and incubated aerobically with shaking at 100 rpm in a 96-well plate in a SPECTROstar Omega plate reader (BMG LABTECH, Ltd, United Kingdom) set to take readings at 5 min intervals. When OD_{600} ∼0.2 was attained, the cultures were treated with 20 μL of 10^9 PFU/mL of the individual phage or various permutations of phage combinations (total MOI ∼10). Efficacy of phages to eliminate the bacterial cultures was ascertained by observing lowest reduced growth impacted by treatment of a phage or phage combination. Low OD_{600} readings reveal effective phage killing and this guided the development of appropriate cocktail for downstream virulence as well as *in vivo* assays.

Optimization of *Salmonella* Infection in *Galleria mellonella* Model

The optimal phage combination developed was tested *in vivo* using the *G. mellonella* larvae *Salmonella* infection model. The larvae were procured, cleaned and prepared as previously described (Nale et al., 2016a). To colonize the larvae with bacterial inocula, cultures of MSG44-S01 (swine), SL1344 (chicken), and T4 (laboratory) strains were prepared in phosphate-buffered-saline (PBS). To do this, a 1:10 dilution of an overnight culture of each strain was prepared in sterile LB broth and incubated aerobically at 37°C until an OD_{600} 0.2 was attained. Cultures were washed three times in PBS by centrifuging at 15,000 *g* for 5 min and resultant pellets re-suspended in PBS each time. The final pellet was resuspended in PBS and diluted to give different bacterial titers and used to colonize *G. mellonella* larvae via oral gavage of 10 μL volumes per larvae using Hamilton pumps as previously described (Nale et al., 2016a).

In order to determine the median lethal dose LD_{50} for each strain in the larva model, a single dose of either, 10^5, 10^4, 10^3, or 10^2 CFU (in 10 μL volumes) of each bacterial inoculum was administered to duplicate groups of four larvae/per group for each bacterial dose. The infected larvae were incubated at 37°C for 24 h. The impact of bacterial colonization on larval survival was ascertained by scoring for live/dead, and the LD_{50} determined by the concentration of *Salmonella* inoculum required to kill approximately half the number of larval populations in each group within the 24 h time frame. Larvae were considered dead when they become inert and turned black in color (Ramarao et al., 2012; Viegas et al., 2013; Nale et al., 2016a). This dose was selected to initiate colonization for each bacterial strain in the *in vivo* phage therapy studies.

To further optimize the infection model, it was vital to ascertain if the phages were stable within the hemolymphs of the larvae to ensure therapeutic efficacy. Therefore, the stability and survival of the phages within the infection model were determined for the proposed maximum experimental time of 72 h. This time was selected as the larvae will only survive for this long at the stated temperature as previously reported (Nale et al., 2016b). Phage survival within the model was done by treating each larva with ∼10^7 PFU of the phage cocktail suspension in 10 μL volume using four larvae per 0, 24, 48, and 72 h time points. The treated insects were incubated as described above. Larval survival, and phages numbers within the hemolymph of each larva and from combined feces of larvae from each time point was done using media above and methods previously described (Nale et al., 2016a).

Phage Therapy Regimens in *G. mellonella Salmonella* Infection Model

Three phage therapy regimens (prophylactic, remedial, and phage/bacterial co-infection), and bacterial- and phage control groups were set up for experimental time points 0, 2, 24, 48, and 72 h for each bacterial strain using four larvae/treatment

regimen/time point (**Table 1**). To initiate colonization, larvae were treated with a 10 μL dose of bacterial inocula used to establish the LD_{50} for each bacterial strain in section "Optimisation of *Salmonella* infection in *Galleria mellonella* model" (10^5 CFU for SL1344, 10^2 CFU for MSG44-S01, and 10^3 CFU for T4) via oral gavage. The three phage therapy regimens were conducted using a single 10 μL dose of the cocktail (at 1:10 bacteria to phage ratio) as previously described for *Clostridium difficile* at time points shown in **Table 1** (Nale et al., 2016a). Briefly, in the bacterial control group (Experimental group 1), larvae were treated with the appropriate dose for each bacterial strain at the 0 h time, and at 2 h treated with sterile LB broth. For the phage/bacteria co-infection regimen (Experimental group 2), the larvae were treated with a combination of the phage cocktail and bacteria at the 0 h and followed by LB at 2 h time point. Larvae in the remedial regimen (Experimental treatment group 3) were treated with bacteria at 0 h before being treated with phage at the 2 h time point. For the prophylactic regimen (Experimental treatment 4), larvae were treated initially with the phage cocktail and after 2 h received a bacterial dose. The final regimen is the phage control group (Experimental group 5), here, larvae were treated with the phage cocktail at the 0 h, and at 2 h treated with LB broth (**Table 1**). After treatments, larvae were incubated at 37°C and remained unfed throughout the experiment (Ramarao et al., 2012; Nale et al., 2016a). At each time points, larvae were scored for survival followed by dissection, and both bacteria and phages were recovered from the hemolymphs on XLD medium using methods previously described (Nale et al., 2016a; Thanki et al., 2019).

Larval survival, and data for CFU and PFU colonization were analyzed using R and GraphPad Prism version 8 (GraphPad Software Inc, United States). To test efficiency of phage treatment regimens, survival data were analyzed using Log-rank (Mantel-Cox) test. CFU data were subjected to Shapiro–Wilk normality test, and each phage treatment was compared with the bacterial control using Mann–Whitney test. Significance was denoted by asterisks, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p = 0.0001$.

TABLE 1 | Time course of phage treatment regimens on *G. mellonella* used in this study.

Experimental groups	Treatments	Time (h)					
		0	2	24	36	48	72
1	Bacterial control	B	LB	-	-	-	-
2	Phage/bacteria Co-infection	P+B	LB	-	-	-	-
3	Remedial regimen	B	P	-	-	-	-
4	Prophylactic regimen	P	B	-	-	-	-
5	Phage control	P	LB	-	-	-	-

Four larvae were used for each time point and treatment regimen. Using Hamilton pump, each larva was treated with 10 μL of phage cocktail (P), bacteria (B), co-culture (P + B), or LB broth (LB). Experiment was repeated three times.

RESULTS

Host Range Properties of Examined Phages on Prevalent *Salmonella* Strains Isolated From Chickens and Pigs

The host range of the phages was evaluated to ensure that they provide suitable coverage against a panel of relevant strains and for the proposed use in an agricultural setting. So, they were first used to challenge prevalent strains of which 10 are commonly found in swine and 23 in poultry. Host range was assessed *in vitro* using host range "spot test" on all the strains, and phage killing assays at MOI of 10 on representative isolates as previously described (Hooton et al., 2011). The strains in the panel represent the top five United Kingdom pig and poultry associated serotypes (**Supplementary Tables S1, S2**).

Among the poultry isolates examined, strains 4–8, 14, and 19–23 were similarly susceptible to all the phages examined as strains were either completely lysed (bacterial strains 5, 7, 8, and 14) or lysed with some resistance on the zones of clearance (strains 4, 6, and 19–23). Although the other bacterial strains 1–3, 9–13, and 16–18 showed variable susceptibilities to the phages, all together the strains were lysed by at least one phage in the collection (**Table 2**). For the pig isolates examined, strains MSG32-S01, MSG52-S01, MSG29-S01, and MSG41-S01 showed the least susceptibility to infection by the phages as these strains were most resistant or showed partial or cloudy lysis with the phages (**Table 3**). On the other hand MSG46-S01, MSG57-S01, MSG44-S01, MSG44-S02, and MSG43-S01 showed most susceptibility to the phages with majority of the strain showing either complete lysis or lysis with some resistant colonies observed on zones of clearance as shown in **Table 3**.

Of the phages tested, STW-77 and SEW-109 showed the most efficacy against both pig and chicken isolates lysing (including cloudy lysis, lysis with resistance and complete lysis) 85% (28 out of 33) of the strains tested, which includes multiple serotypes. The remaining phages showed similar lytic activity against most of the strains covering between 60 to 70% of the strains tested (**Tables 2**, **3**).

Activity of Phages on Growth of *Salmonella in vitro*

To develop a maximally effective phage cocktail, we examined and selected phages with the highest host range activity. Therefore, phages ST-W77 and SE-W109 were selected because of their wide host range activity on the swine and chicken isolates examined (**Tables 2**, **3**). Phage SPFM17 was also included as it was the phage with the widest coverage on the chicken isolates that can also lyse MSG46-S01 and MSG32-S01, which were the swine isolates with the least susceptibility to other phages. Therefore, phage SPFM17 in combination with phages ST-W77 and SE-W109 can lyse over 90% of the bacterial strains from swine and poultry isolates tested (**Table 3**).

To examine the complementation effects of the selected phages, all optimizations of cocktail development using the three phages were conducted on the phage propagating host, SL1344, which is also a chicken isolate. When the individual phages were

TABLE 2 | Host range activity of the 22 phages against 23 prevalent poultry isolates examined in this study.

Phages	Chicken bacterial strains																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
SPFM9																							
SPFM11																							
SPFM17																							
SPFM4																							
SPFM2																							
SPFM19																							
SPFM14																							
SPFM10																							
SPFM12																							
SPFM13																							
SPFM20																							
SPFM1																							
SPFM3																							
SPFM16																							
SPFM15																							
SPFM7																							
SPFM6																							
SPFM8																							
SPFM21																							
SPFM5																							
ST-W77																							
SF.-W109																							

Confluent bacterial cultures of representative Salmonella chicken strains were prepared in 4 mL of 0.7% LB agar medium. Approximately, 10 μL of 10^8 PFU/mL of phage lysate was applied to the lawn and zones of lysis were observed after incubation at 37°C aerobically for 18–24 h. Key: red = no infection, mustard = cloudy lysis, green = lysis with some resistant colonies observed on zones of clearance, and blue = complete lysis with no resistance.

added to the growing culture at OD_{600} 0.2 (at 100 min, indicated with a green arrow), the growth of the bacterium decreased at 100 min after adding phage for all the individual phages and this reduction was maintained for an additional 150 min (for phage ST-W77) and 80 min (for phages SE-W109 and SPFM17) post phage exposure (**Figure 1A**). However, after ∼200 min (for SPFM17), 500 min (for ST-W77), and 600 min (for SE-W109) post phage treatment, bacterial regrowth was observed (**Figure 1A**). Next, the individual phage lysates were combined at equal proportions to form a cocktail with the same overall MOI as when phages were used individually, and this was used to infect SL1344 culture at OD_{600} 0.2 at the same growing time of 100 min. The bacterial growth continued to progress for an additional 300 min but then decreased to OD_{600} 0.1 at 550 min post phage treatment (for SL1344) and this level remained consistent until the end of the experiment (**Figure 1B**). Resistant strains (five clones for each phage treatment) were isolated and challenged with other phages in the mix. It was observed that phage resistant strains produced by one phage was lysed by one or two other candidate phage for the cocktail development (**Supplementary Table S3**).

Having ascertained the impact of the individual and the three-phage cocktail on cultures of the propagating host, SL1344, we then tested the activity of the cocktail on a swine isolate MSG44-S01, which is fully susceptible to the three phages, and a laboratory strain T4, which is routinely used for most of our *Salmonella* work. The phage cocktail completely eliminated the two additional bacterial strains beyond limit of detection ∼700 min post phage cocktail exposure. This observation remained consistent till the end of the experimental time and no re-growth was observed (**Figure 1B**).

Stability of *Salmonella* Phages in *G. mellonella*, and Establishment of Infective Doses of Pig, Chicken and Laboratory Reference Isolates Examined

Having established the efficacy of the cocktail *in vitro* on the representative chicken, swine and on the laboratory test strains, we then tested the lysis activity *in vivo* using the *G. mellonella* larvae. The stability of the phages within the model was ascertained by establishing they were stable and recoverable within the guts and feces of the larvae (Nale et al., 2016a). Our data showed that there was no significant lose in phage titer within the gut of the larvae throughout the 72 h experimental period. Similarly, phages were shed in the feces, albeit a ∼4 log10 PFU/larva reduction was observed 24 h post-exposure but this level remained consistent over the subsequent 48 and 72 time points (**Supplementary Figure S1A**). Because the bacterial cultures were suspended in PBS before colonizing the larva, we confirmed that the phages were also stable in this buffer as well as no significant loss in titer was

TABLE 3 | Host range activity of the 22 phages on 10 prevalent swine isolates examined in this study.

Phages	Swine bacterial strains									
	MSG41-SOI	MSG46-SOI	MSG52-SOI	MSG57-SOI	MSG44-SOI	MSG44-S02	MSG26-SOI	MSG29-SOI	MSG32-SOI	MSG43-SOI
SPFM9										
SPFM11										
SPFM17										
SPFM4										
SPFM2										
SPFM19										
SPFM14										
SPFM10										
SPFM12										
SPFM13										
SPFM20										
SPFM1										
SPFM3										
SPFM16										
S0FM15										
SPFM7										
SPFM6										
SPFM8										
SPFM21										
SPFM5										
ST-W77										
SE-W109										

Confluent bacterial cultures of representative Salmonella swine strains were prepared in 4 mL of 0.7% LB agar medium. Approximately, 10 μL of 10^8 PFU/mL of phage lysate was applied to the lawn and zones of lysis were observed after incubation at 37°C aerobically for 18–24 h. Key: red = no infection, mustard = cloudy lysis, green = lysis with resistance on the zones of clearance, and blue = complete lysis with no resistance.

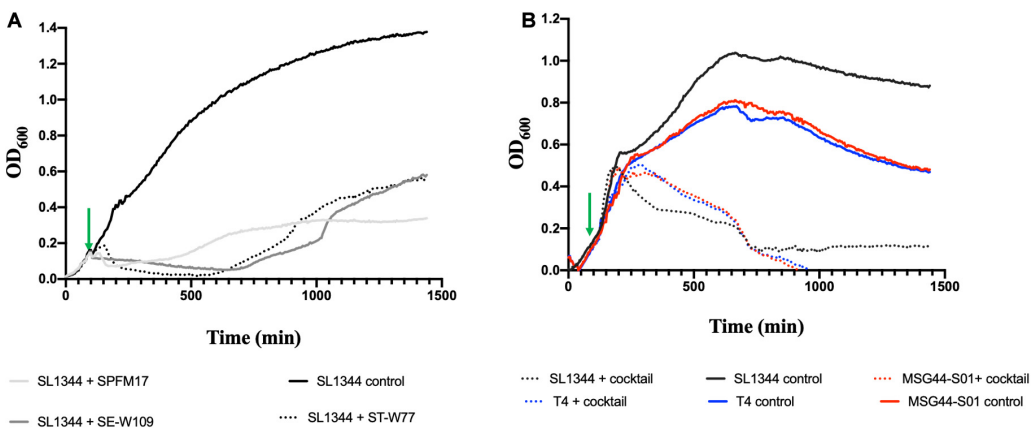

FIGURE 1 | Lysis activity of candidate phages used for cocktail development on bacterial growth of representative swine, poultry and laboratory strains examined in this study. Virulence assay was conducted in 200 μL volumes in SPECTROstar Omega plate reader, containing phage/bacteria at MOI of 10. **(A)** show individual phage killing on SL1344 and **(B)** impact of three-phage cocktail on cultures of chicken SL1344 (black line), swine MGG44-S01 (red line), and laboratory reference T4 strains (blue line). Green arrows represent points when phages were added. Data was analyzed using GraphPad Prism 8.

observed after resuspending the phages in the buffer for an hour (**Supplementary Figure S1B**). In addition to determining the phage stability within the larvae and PBS during the *in vivo* model optimization, we further determined the individual strains LD_{50} within 24 h to determine the bacterial numbers needed to cause colonization and to cause death in ∼50% of the larval population within this time frame. This is essential to enable various therapeutic regimens to be tested with the 72 h time frame (Nale et al., 2016a). It was observed that the LD_{50} values were variable for the three strains tested with the lowest being 10^2 CFU/larva for the swine strain MSG44-S01, which is the swine isolate. However, for T4 and SL1344, 10^3 and

10^5/larva, respectively, were required to exert relative LD_{50} effect as in MSG44-S01.

Impact of Phage Treatment on *G. mellonella* Infected With Various *Salmonella* Isolates

Having fully developed the phage cocktail *in vitro* and optimized the *G. mellonella Salmonella* infection model, the efficacy of the phage cocktail was then tested on larvae colonized with the chicken SL1344, swine MSG44-S01 and laboratory T4 representative *Salmonella* strains. Colonization was established using a single dose of the optimized bacterial culture and followed by various therapeutic phage regimens to determine which treatment would reduce *Salmonella* colonization and enhance survival of the larvae the most.

Efficacy of Phage Treatment on *G. mellonella* Infected With Chicken Isolate, SL1344

For the SL1344 chicken strain, larval group treated prophylactically survived throughout the experimental time, which is significant compared to the bacterial control group ($p < 0.0001$). Although larval group treated with a co-culture of the phage and bacteria survived until the 36 h, 10% of infected and treated larvae died by the 48th hour but the remaining larvae survived until the end of the experiment. The co-infection regimen is not as efficient as the prophylaxis ($p < 0.001$). The treatment group with the least survival was exhibited by the remedial group, where, ∼95% survived within the first 24 h, and this is not significantly different compared to the control bacterial groups. Continual reduction was observed through the course of the time points, with 86, 60, and 10% survival at the 36th, 48th, and 72nd hour, respectively. The bacterial control group treated with cultures of SL1344 and no phage also showed gradual decrease in survival from 86% at the 24th hour to all larvae dead at the 72nd hour (**Figure 2A**).

With respect to the colonization of SL1344 within the insects, we observed complete eradication of the bacteria within 24 and 72 h post treatment in the prophylactic and phage bacterial co-culture treatments, respectively. Colonization in the remedial regimen gradually decreased from $\sim 10^4$ CFU/larva to an undetectable level at the 24th hour, however, bacterial regrowth was observed after 36 h when up to 10^4 CFU/larva was observed to 10^7 CFU/larva at the 48 and 72 h times (**Figure 2B**). When compared with the bacterial control group, prophylaxis regimen was more effective at reducing colonization of this strain ($p < 0.05$) but no significant difference was observed with the co-culture and remedial regimens.

Regarding phage counts, the phage control and prophylactic groups showed a steady level until 48 h followed by a 2 log PFU/larva in the phage control group and a 3 log PFU/larva reductions of phage counts in the prophylactic group at the 72 h time. The phage bacterial co-culture group showed steady phage increase up to $\sim 10^5$ PFU/larva at the 72 h. In the remedial regimen 10^5 PFU/larva of phages were recovered at 2 h time but phage numbers later dropped to 10^3 PFU/larva from the 36th hour to the end of the experimental time of 72 h (**Figure 2C**).

Effect of Phage Treatment on *G. mellonella* Infected With Swine Isolated, MSG44-S01

For the swine strain MSG44-S01, larvae in the phage control, prophylactic and the co-culture groups all survived throughout the experiment, and both regimens are significant compared to the bacterial control groups ($p < 0.05$). In the remedial regimen and bacterial control, only 13% death at the 48th hour was observed in both groups, which is not significant compared to the bacterial control groups. Although this level remained stable till the 72nd hour time point for the remedial regimen, only 20% larval survival was observed in the bacterial control larval group at this time (**Figure 3A**).

With the swine bacterial strain, colonization in the bacterial control group progressed from 10^2 CFU/larva at the beginning of the *in vivo* assay to 10^5 CFU/larva at the end of the experimental 72nd hour time point. Comparing colonization in larvae within the therapy regimens, it was observed that treating the insect with the phage cocktail prophylactically 2 h before exposing them to the bacteria resulted in the complete prevention of colonization as assessed at the 24th hour. Similarly, administering a phage and bacterial mixture resulted in the eradication of the bacteria at the 36th hour time point, where bacteria were undetectable in the larvae. Consistent with the survival data, both prophylaxis and co-culture regimens significantly eradicated the bacteria from the larvae compared to the bacterial control ($p < 0.01$). In contrast to the other treatments, the remedial regimen was not very effective at eradicating this strain from the larvae as variable colonization levels ranging from undetectable level to 10^5 CFU/larvae was observed in some of the larvae within this treatment group (**Figure 3B**). This treatment was not significant compared to the bacterial control group for this strain.

The phage level remained relatively consistent as observed with SL1344 strain, although a 2 log PFU/larva was lost at the 72nd hour in the phage control group, and this pattern is similar in the prophylactic group for this strain. Phage recovery in the phage/bacterial co-infection group increased to 10^4 PFU/larva at the 36th hour but decreased from the 48th till the 72nd hour time point with 10^3 PFU/larva recovered. In the remedial regimen with this strain, less phages were recovered and titer decreased from 10^3 PFU/larva to 10^2 PFU/larva but increased to 10^3 PFU/larva at the 36th hour but later dropped to 10^2 PFU/larvae, however, in other larva phages were not detected in this regimen (**Figure 3C**).

Impact of Phage Treatment on *G. mellonella* Infected With Laboratory Strain, T4

Colonizing the larvae with our reference laboratory strain, T4 and treating with the optimized phage cocktail showed 100% survival among phage and prophylaxis treated larval groups, which is significant compared to the bacterial control group ($p < 0.0001$; **Figure 4A**). The efficacy of this regimen on this strain is consistent with observations of this treatment regimen in both the chicken and swine isolates shown in **Figures 2A**, **3A**, respectively. With the co-infection regimen, 83% of the larvae survived at 36 h but survival dropped to 72% for the laboratory reference strain at 48 h, and this level remined consistent till the end of the experiment (72 h). This is significant when

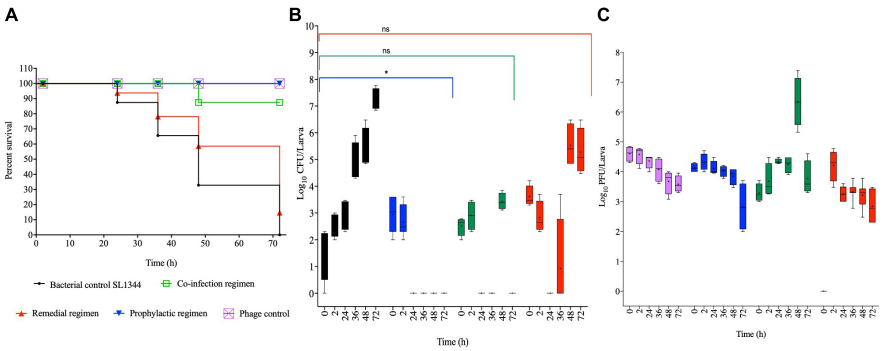

FIGURE 2 | Impact of phage therapy on *G. mellonella* colonized with chicken isolate SL1344. Larvae were colonized with 10^5 CFU each in 10 μL via oral gavage. Phage therapy regimens were conducted using 10^6 PFU/larva. **(A)** show survival, **(B)** *Salmonella* colonization, and **(C)** Phage recovery at various time-points for each treatment-Control bacteria (Black), Co-infection (Green), Remedial (Red), Prophylactic (blue) and Control phage (Purple) and Prophylactic (Blue) lines/bars. Four larvae were used for treatment and timepoint. Experiment was repeated thrice and analyzed using Shapiro–Wilk normality test on R. Each phage treatment regimen was tested against SL1344 control using Mann–Whitney test on GraphPad Prism 8. ns = No significance, *significance at $p < 0.05$.

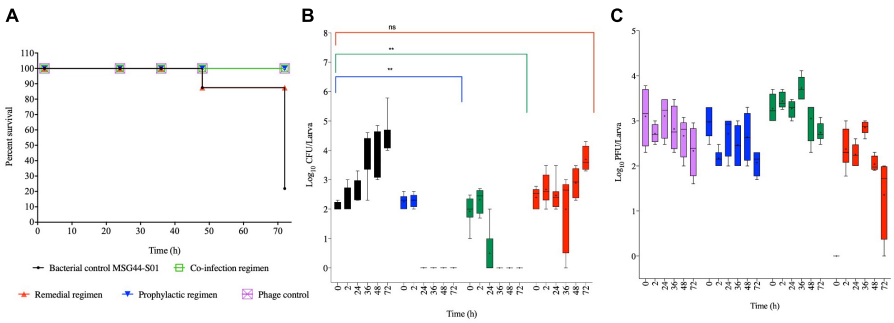

FIGURE 3 | Impact of phage therapy on *G. mellonella* colonized with swine isolate MSG44-S01. Larvae were colonized with 10^2 CFU each in 10 μL via oral gavage. Phage therapy regimens were conducted using 10^3 PFU/larva. **(A)** show survival, **(B)** *Salmonella* colonization, and **(C)** Phage recovery at various time-points for each treatment-Control bacteria (Black), Co-infection (Green), Remedial (Red), Prophylactic (blue) and Control phage (Purple) and Prophylactic (Blue) lines/bars. Four larvae were used for treatment and timepoint. Experiment was repeated thrice and analyzed using Shapiro–Wilk normality test on R. Each phage treatment regimen was tested against MSG44-S01 control using Mann–Whitney test on GraphPad Prism 8. ns = No significance, **significance at $p < 0.01$.

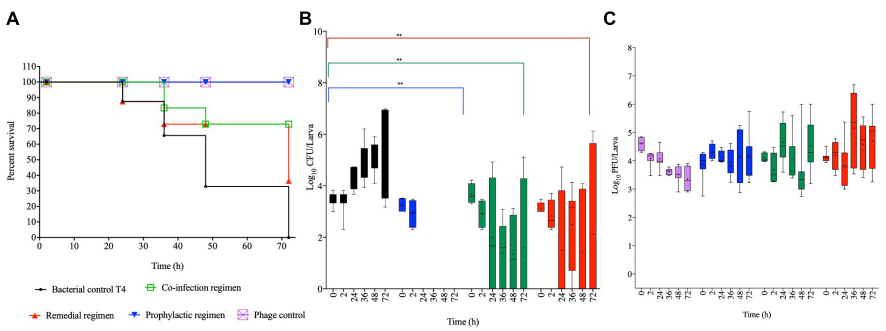

FIGURE 4 | Impact of phage therapy on *G. mellonella* colonized with laboratory isolate T4. Larvae were colonized with 10^3 CFU each in 10 μL via oral gavage. Phage therapy regimens were conducted using 10^4 PFU/larva. **(A)** show survival, **(B)** *Salmonella* colonization, and **(C)** Phage recovery at various time-points for each treatment-Control bacteria (Black), Co-infection (Green), Remedial (Red), Prophylactic (blue) and Control phage (Purple) and Prophylactic (Blue) lines/bars. Four larvae were used for treatment and timepoint. Experiment was repeated thrice and analyzed using Shapiro–Wilk normality test on R. Each phage treatment regimen was tested against T4 control using Mann–Whitney test on GraphPad Prism 8. ns = No significance, **significance at $p < 0.01$.

compared to the bacterial control ($p < 0.01$) but not as efficient as the prophylaxis regimen shown above. The remedial regimen revealed lowest survival with 85% survival at 24 h but this declined to 73% at both 36 h and 48 h, and finally to 36% at 72 h. Although the remedial regimen is the least effective regimen for this strain it is still significant compared to the bacterial control

group ($p < 0.05$). The bacterial control showed gradual decline in survival from 87% survival at the 24th hour to complete larval death at the 72nd hour (**Figure 4A**).

Data on colonization indicated that T4 strain also colonized the larvae with $\sim 10^3$ CFU/larvae observed at the beginning of the experiment to 10^7 CFU/larva at the end of 72 h time point. As the pattern in the other two bacterial strains, after 2 h, the bacteria were completely eradicated and were undetected in the larvae in the prophylactic regimen. The co-infection and remedial regimens showed similar colonization levels with starting bacterial count of 10^{3-4} CFU/larva to variable levels at the subsequent time points ranging from undetectable level in some insects to 10^6 CFU/larva in some at the end of the experiment. All the individual phage therapy regimens significantly eradicated the T4 strain compared to the control ($p < 0.01$; **Figure 4B**).

Results for phage counts showed higher PFU numbers within the larvae in the remedial treatment group compared to larvae in the other two phage treatment regimens. In all the phage treatment groups, phage counts within the larvae ranged from 10^{4-5} PFU/mL starting phage level to 10^6 PFU/mL in some of the insects at the end of the experimental time was observed (**Figure 4C**).

DISCUSSION

Salmonella infection arising from eating contaminated food products remains a major concern to human health with greater percent of cases resulting in mild to severe intestinal gastroenteritis and fatality in others (Sockett and Roberts, 1991; Majowicz et al., 2010). As a result, a number of trade restrictions are introduced in cases where there is contamination with prevalent serotypes of *S.* Enteritidis and *S.* Typhimurium leading to major loss of income to farmers and producers due to rejection of substandard contaminated animal products (Majowicz et al., 2010; Kirk et al., 2015; EFSA Panel on Biological Hazards et al., 2019). Although antibiotics are useful in controlling the infection in both humans and animals, many of the bacterial strains are becoming resistant to routinely used antibiotics leading to treatment failure and disease outbreaks (O'Neil, 2014; Fong et al., 2020). As the identification and development of new antibiotics is slow and difficult, the associated economic and social loss highlight the pressing need to develop alternative more effective therapeutics for this infection (O'Neil, 2014; Romero-Calle et al., 2019). Here, data to support a viable alternative way to control infection in humans is presented. This research focuses on the development of a highly effective *Salmonella* phage cocktail *in vitro* and showing its efficacy in *G. mellonella Salmonella* infection model using various regimens. The data presented here will inform the application of the optimized phage cocktail to effectively prevent or reduce bacterial colonization in animals, thus breaking the cycle of infection and producing safer animal products in the market as previously shown (Wall et al., 2010; Nabil et al., 2018).

The choice of phage therapy approach to control *Salmonella* colonization in animals as proposed in this study has great inherent advantages over conventional antibiotic use. Microbes thrive easily where favorable pH, temperature, moisture and nutrients are present. However, because at ambient conditions or higher temperatures antibiotics efficacy diminishes with time, multiple applications are needed to sustain an effective dose to control a growing bacterial population (Mackowiak et al., 1982; Paterson et al., 2016). In contrast, phages are biological entities, and have been shown to be more stable in various pHs, biotic environments and in ambient conditions than antibiotics (Ahmadi et al., 2017; Sommer et al., 2019). In addition to stability, phages replicate and produce increasing infective particles in the presence of target bacterial pathogen, hence ensuring continuous dosage supply (auto-dosing) of anti-infectives at infection sites (Jończyk et al., 2011; Loc-Carrillo and Abedon, 2011). Furthermore, phages can selectively remove targeted bacteria but exclude other microbial commensals in the niche leaving them unharmed, and this may particularly help animal gut-health, thus producing better quality animal products (Moye et al., 2018; Divya Ganeshan and Hosseinidoust, 2019). Since phages are generally regarded as safe, they are excellent candidates to control *Salmonella* colonization and biofilm development in various ready to eat foods, milk, pigs, and chickens to reduce *Salmonella* colonization (Wall et al., 2010; Huang et al., 2018; Nabil et al., 2018; Islam et al., 2019).

Pertinent to controlling *Salmonella* infection in animals, most previous studies have focused on isolating phages from the environment, testing the activity of individual phages and developing various combinations of phage cocktails with the aim of reducing the bacterial numbers *in vitro* and *in vivo* (Pereira et al., 2016; Islam et al., 2019; Phothaworn et al., 2020). The challenge, however, has been the difficulties of isolating therapeutic phages with acceptable genomic properties, host range coverage and the translation of observed *in vitro* activity to *in vivo* applications in target animals (Nilsson, 2014; Hyman, 2019). All phages examined here are known to be obligately lytic and do not encode undesirable genes expected in a therapeutic phage product (Thanki et al., 2019; Phothaworn et al., 2020). In addition to the genome contents, the phages have been shown to have a wide host range activity on various poultry and swine related *Salmonella* serotypes, thus are excellent candidates for therapeutic purposes in animals (Thanki et al., 2019; Phothaworn et al., 2020). To further ensure that the phages can target the correct strains examined here, they were further challenged with prevalent *Salmonella* serotype strains currently causing infection in pigs and poultry in the United Kingdom, as well as in humans globally (Majowicz et al., 2010; EFSA Panel on Biological Hazards et al., 2019). Despite their variable lysis efficacies on the strains examined in this study, together the phages were able to lyse at least one of the bacterial strains, including the monophasic *S.* Typhimurium strain associated with micro evolution of multi-drug resistance and epidemiologic success (Hugas and Beloeil, 2014; Petrovska et al., 2016; Branchu et al., 2019; Campos et al., 2019; Petsong et al., 2019; Tassinari et al., 2019). These observations concurred with other previous studies which reported phages targeting MDR *Salmonella* strains (Atterbury et al., 2007; Hooton et al., 2011; Jung et al., 2017).

The three-phage cocktail developed here comprised of two myoviruses (SPFM17 and ST-W77) and a siphovirus (SE-W109), indicating that being of diverse morphologies, they may target

diverse bacterial host strains resulting to a broad-spectrum cocktail. In synergy to this, our data showed that the individual phages in the mix have complementary contributory target coverage and together lysed 100% of the tested pig isolates, 99.95% of the chicken isolates and combined ∼99.97% of the total serotype strains examined. This suggests that the phages may encode different tail fiber proteins which enabled them to target different receptors on the different host bacteria (Drulis-Kawa et al., 2012). This feature may confer advantage for their therapeutic use as a cocktail, but further work is required to determine this within the genomes of our phage mix. Optimizing our cocktail with diverse phage morphologies concurred with other findings, however, it some cases, single or unknown morphologies were used to construct an effective cocktail (Hooton et al., 2011; Costa et al., 2019; Petsong et al., 2019; Stone et al., 2019). The observed host range coverage of the cocktail spanning various pigs and poultry isolates has been shown in other reports and this further support the prospective multi-purpose application of the cocktail to treat these animals (Petsong et al., 2019). Thus, having both therapeutic and economical advantage to be used in swine and poultry industries.

The phage cocktail we developed has the required host range coverage and has clearly shown efficacy at significantly eliminating the examined bacterial cultures than individual phage treatments *in vitro* using an MOI of 10 (Hooton et al., 2011). For the pig and laboratory reference strains, the phage cocktail completely eliminated the bacterial cultures below the limit of detection, although reduced activity was observed with the chicken isolate. Although enhanced clearance of bacteria using cocktail was reported in previous work on *Salmonella,* other phage cocktails were shown to be no superior to individual phage treatments due to continuous resistance development after treatment in the mix (Hooton et al., 2011; Costa et al., 2019). The observed effective clearance by the optimized cocktail developed here was achieved by a complementation effect, where one phage resistant strain is lysed by another wild-type phage in the mix. This activity concurred with a previous report on *C. difficile*, where resistant/lysogenic strains emanating from one phage infection were efficiently lysed by another phage in the cocktail (Nale et al., 2016b). Although various phage cocktails have been developed for *Salmonella*, this is the first time that this kind of interaction is reported on this species.

The next step in our project was to translate the knowledge obtained on the phage activity *in vitro* into a potential application *in vivo* and to determine which therapeutic regimen would be best in eliminating *Salmonella* in *G. mellonella* model. Therefore, to develop the model for *Salmonella* infection it was essential to begin by optimizing the LD_{50} for each of our test bacterial strain to ensure we have sufficient bacterial load to cause relatively equal effect across the strains tested. Our observation showed that a higher bacterial load of 10^5 CFU/larva of the chicken strain SL1344 was required to exert comparable LD_{50} effect compared to lower doses of 10^3 CFU/larva and 10^2 CFU/larva for the swine and laboratory reference strain. Our observation on the SL1344 chicken isolate concurred with previous *Salmonella* infection work on *G. mellonella* which showed that any dose above 10^5 CFU/larvae caused death in all larvae within 24 h (Viegas et al., 2013). Except that in our studies we observed ∼50% death in the larvae within this timeframe and this may be attributed to differences in *G. mellonella* type or method of administration. In our study, larvae were colonized via oral gavage while in the previous work colonization was achieved via proleg injection (Viegas et al., 2013).

Comparing treatment regimens, it was clear that prophylaxis was more effective at controlling colonization of all the *Salmonella* strains tested compared to remedial or co-infection with phage and bacteria. This observation is in agreement with *Salmonella* phage treatment in quals and phage therapy studies conducted in the larvae using other pathogens such as *C. difficile* and *Pseudomonas aeruginosa* (Beeton et al., 2015; Ahmadi et al., 2016; Nale et al., 2016a). This observation with *Salmonella* could be attributed to the fact that pre-treating the larvae with the phages for 2 h provided sufficient time for the phage to adapt to the gut environment of the larva as shown in the stability assay, and hence were able to effectively kill the bacteria when administered (Nale et al., 2016a). The other regimens (remedial and co-infection) did not do as well as the prophylaxis and this may be attributed to the ability of *Salmonella* to get intra-cellularized and this may reduce the efficacy of the phages (Diacovich et al., 2017).

CONCLUSION AND FUTURE WORK

Gastro-enteritis caused by *Salmonella* is a major health challenge. The infection is contracted via eating contaminated animal products. Antibiotics are helpful but bacteria are becoming resistant to many front-line antibiotics, hence viable alternative control is urgently needed to reduce the health and economical loss. Here, we reported an approach to the development of an effective therapy using phages to stop infection in animals before products are processed for consumption. To do this we first optimized a broad host-range phage cocktail, which cleared *Salmonella* efficiently *in vitro* and showed that prophylactic treatment regimen is the most effective approach to control the infection in *G. mellonella* larva model. The data presented here provides a robust pre-livestock data to support the translation of this cocktail to effectively treat the infection in chickens and pigs. Work is currently ongoing to formulate the phages into pH- and heat-stable powders, and incorporated into feeds and used to control *Salmonella* infection in the target animals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

GV and JN conducted the *in vitro* assays. JN and VL conducted the *in vivo* work. AT and PP isolated the phages. MAO, MFA, and AG isolated the chicken and swine isolates. JN, GV, VL, and AT drafted the manuscript. JN and GV analyzed the

data. JN, MC, EG, SK, DM, GV, MAO, MFA, AG, and PT conceived and designed the experiments. All authors edited and 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: https://www.frontiersin.org/articles/10.3389/fmicb.2020.609955/full#supplementary-material

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Phages Bind to Vegetative and Spore Forms of *Paenibacillus larvae* and to Vegetative *Brevibacillus laterosporus*

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Paenibacillus larvae is the causative agent of American Foulbrood (AFB), the most destructive bacterial infection in honeybees. Even antibiotic-sensitive strains of *P. larvae* can produce recurrent AFB months to weeks post-antibiotic treatment due to the survival of bacterial spores. Recently, phages that infect *P. larvae* have been shown to effectively combat AFB in the field. Here, we present evidence that phages not only bind to vegetative *P. larvae* but also bind to *P. larvae* spores. Spore binding was observed in the results of three specific experiments: (1) bacteria counted by flow cytometry generated quantitative data of FITC-labeled phages that were bound to vegetative bacteria as well as those bound to spores, (2) electron microscopy captured images of phages bound to the surface of spores in both horizontal and vertical positions, and (3) phages incubated with *P. larvae* spores bound to the spores and created plaques in vegetative bacteria under conditions not conducive to spore activation, indicating that binding to spores is reversible and that the phages are still active. Identification of phages with reversible spore-binding capability for use in phage therapy may improve treatment of sporulating bacterial infections.

Keywords: American Foulbrood, bacteriophage, phage therapy, *Paenibacillus larvae*, bacterial spores, phage binding, spore binding, Brady Binding Assay

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INTRODUCTION

The spore forming bacterium *Paenibacillus larvae* causes American Foulbrood (AFB) in honeybees. An AFB outbreak begins when there are too many *P. larvae* spores in the honey crop of a nurse bee to be cleared naturally and spores get passed to honeybee larvae (Lindstrom et al., 2008). In the larval intestinal tract, *P. larvae* spores germinate to become vegetative bacteria capable of producing toxins that liquefy the honeybee larvae (Djukic et al., 2014). The resulting degraded larvae become laden with *P. larvae* spores which are then tracked to other larvae in the hive by nurse honeybees (Ratnieks, 1992). The disease spreads quickly within a hive, taking just several days from initial infection to decimation of a colony (Chantawannakul and Dancer, 2001; Genersch, 2010). After the colony collapses, other colonies may rob the contaminated honey and further spread AFB via spores with an 80% transmission rate during an outbreak in an apiary (Brady et al., 2017).

Due to their host specificity, phages can target and destroy pathogenic bacteria while leaving commensal bacteria alone because, most typically, a phage is limited to a narrow range of strains of the same species (Merrill et al., 2014; Cresawn et al., 2015; Brady et al., 2017; Hatfull, 2018). The

narrow host specificity often requires the use of a phage cocktail (more than one phage) in order to target different strains of the same species (Brady et al., 2017; Dedrick et al., 2019; Corbellino et al., 2020). Phage therapy with an appropriate cocktail is an effective treatment option for active AFB infections, demonstrating a 100% recovery and prevention rate in treated hives (Brady et al., 2017). Furthermore, hives treated with *P. larvae* phages experienced no reinfection of AFB, which may indicate that the phage treatment also neutralized *P. larvae* spores (Brady et al., 2017).

We hypothesize that the ability of *P. larvae* phages to prevent reinfection of AFB as observed by Brady et al. (2017), is due to the *P. larvae* phages' ability to bind to bacterial spores. To obtain supportive data for this hypothesis, we determined to identify whether *P. larvae* phages could, in fact, bind to spores and whether or not such phages would still be capable of infection. We report that flow cytometry data indicated statistical significance in support of spore-binding, that electron micrographs captured phages attached to the side of spores, and that the phages that bind to *P. larvae* spores were still active and could subsequently infect vegetative bacteria. Another finding of our phage-binding studies was that *P. larvae* phages also bound to vegetative *Brevibacillus laterosporus* at a statistically higher amount than to another unrelated bacterium. *B. laterosporus* is a Firmicutes bacterium also found in honeybees. The *P. larvae* phage used in our studies could not productively infect *B. laterosporus*, so binding of the phages to *B. laterosporus* may indicate a survival mechanism for phages between active AFB infections.

MATERIALS AND METHODS

Spore Generation and Extraction

Overnight cultures of *P. larvae* ATCC 9545 grown in 1/2 × liquid porcine brain heart infusion (PBHI) (Acumedia, Lansing, MI, United States) media were grown in a shaking incubator at 37°C. The optical density of the culture at 600 nm was used to estimate the number of cells per milliliter as we have reported previously (Brady et al., 2017). A total of approximately 10^4 bacterial cells were spread onto tryptic soy agar plates with glass beads and allowed to incubate at 37°C and 5% CO_2 for 8 days. Incubated plates were doused with five ml of cold sterile ddH_2O and allowed to sit for 15 min. Colonies on the plates were gently scraped off the plate and into suspension with sterile loops. The suspensions from eight plates were combined into a 50 ml tube and centrifuged at 12,000 × *g* for 20 min. Supernatant was poured off and the pellet was resuspended in 40 ml of sterile ddH_2O and centrifuged again as a wash step. The pellet was washed two more times. After the last wash step, the pellet was resuspended in 80% EtOH.

Spores were removed from the ethanol immediately prior to any experiment. Spores suspended in ethanol were centrifuged at 12,000 × *g* for 5 min to pellet the spores and the supernatant containing alcohol and the lysed debris from dead vegetative cells were discarded with the supernatant. The pellet was washed three more times using sterile 1/2 × PBHI broth and then suspended to a concentration of 10^4 colony forming units (CFU) per ml. Spore purity was also confirmed using the Schaeffer-Fulton staining method: briefly, samples were heat fixed, stained with 5% malachite green for 5 min over heat, and counterstained with 0.2% safranin (Schaeffer and Fulton, 1933). Spore viability and purity tests were done with samples in triplicate. The malachite green and safranin staining was done to verify spore presence and identify any residual vegetative bacteria in the sample. At least 100 cells from each sample were observed and counts taken for the number of spores, spores-in-mother cells, and vegetative cells.

Phage Generation

Phages specific for *P. larvae* were previously isolated and confirmed to infect and lyse only *P. larvae* (Brady et al., 2017; Stamereilers et al., 2018) and not *B. laterosporus* (Brady et al., 2017; Berg et al., 2018; Brady et al., 2018). Phages were tested on field isolates of *P. larvae*. Each field isolate for phage characterization was identified as *P. larvae* by confirming gram test, catalase test, 16s sequencing, and by KAT PCR and ERIC PCR. Primers and citations for these methods are indicated in **Table 1**. Protocols are published in our previous works (Brady et al., 2017; Berg et al., 2018; Brady et al., 2018). The phage, PL.Ph-1, was previously isolated from a dead feral beehive in Utah that appeared to have died from AFB. PL.Ph-1 forms lytic plaques on the laboratory standard *P. larvae*, ATCC 9545, as well as on 55 of 59 verified field isolates of *P. larvae* as previously reported (Brady et al., 2017). Phage lysate of PL.Ph-1 [the phage also indicated as phage 1 in Brady et al. (2017)] was prepared for these studies by reconstituting a freezer stock sample of the phage in 500 μL of overnight *P. larvae* and plating the solution in 1/2 × PBHI top agar and left to incubate at 37°C. After 24 h, visible plaques were selected from the plate, suspended in 25 ml of 1/2 × PBHI broth to which was added 1×10^6 CFU of *P. larvae* and incubated, shaking at 37°C. After 16 h the lysate was filtered through a 0.22 μM filter (VWR, Radnor, PA, United States). Lysate concentration was determined using standard titration techniques as previously described to determine plaque forming units (pfu) per ml (Brady et al., 2017). Titered lysate of PL.Ph-1 was used for all studies in this paper.

Phage Binding Detection by FITC Stain and Flow Cytometry

Unconjugated FITC was added to a high titer phage lysate (10^{11} pfu) suspended in 1× HEPES solution (pH 7.4) to obtain a concentration of 31.25 μg/ml FITC and was allowed to incubate for 1 h. The high titer lysate was ultracentrifuged at 25,000 × *g* for 1 h to pellet the phages. The supernatant containing unbound FITC was poured off and the pellet resuspended in HEPES solution to a FITC concentration of 15.625 μg/ml to avoid background staining of bacteria as indicated in the results.

For flow cytometry analysis, bacterial samples were loaded into a 96-well plate containing approximately 5×10^4 CFU in each well. Each well received 200 μL of FITC-labeled phages. Cell fluorescence was measured by a Cytoflex S flow cytometer and a minimum of 50,000 cells were counted per sample. Three experiments were run on separate weeks with three replicates for each sample in each experiment.

TABLE 1 | Primers for PCR identification of *P. larvae* field isolates used to challenge *P. larvae* phages.

Primer	Sequence	Results	References
16s 27-forward	5′-AGAGTTTGATCMTGGCTCAG-3′	16s rRNA universal primer	Lane, 1991
16s 907-reverse	5′-CCGTCAATTCMTTTRAGTTT-3′		
KAT-Forward	5′-ACAAACACTGGACCCGATCTAC-3′	Generates a band only if *P. larvae* ERIC-1 or ERIC-2 genotype	Alippi et al., 2004
KAT-Reverse	5′-CCGCCTTCTTCATATCTCCC-3′		
ERIC1-Forward	5′-ATGTAAGCTCCTGGGGATTCAC-3′	Generates a series of bands used to identify *P. larvae* types	Versalovic et al., 1994
ERIC1-Reverse	5′-AAGTAAGTGACTGGGGTGAGCG-3′		

Flow Cytometry Data Analysis

Beckman Coulter CytExpert software was used to analyze the flow cytometry data collected on the Beckman Coulter Cytoflex S flow cytometer. Three gates were individually set using unstained samples of each bacterial type using channels for Forward Scatter (FSC), Side Scatter (SSC) and FITC. The gates were set on FSC × SSC to exclude debris, FSC-HxFSC-A to isolate single cells, and on the FITC channel to identify positive or negative samples.

Phage Binding Detection by Electron Microscopy

Vegetative *P. larvae* and *P. larvae* spores (5×10^5 CFU) were resuspended in 1 ml of 3×10^9 pfu/ml high titer lysate and allowed to incubate for 1 h. The phage-treated spores were pelleted at 8,000 rpm for 6 min. The supernatant was poured off and the pellet was resuspended in 40 μL of 1× HEPES solution.

Phage/spore samples were incubated with formvar coated copper grids for 60 s and then incubated with 50 μL of 2% uranyl acetate (pH 7) for 60 s. Moisture was wicked away from the grids and then allowed to air dry prior to imaging. Electron micrographs were taken by the BYU Microscopy Center on a Verios STEM machine (Abràmoff et al., 2004).

Phage Binding and Viability Detection Using the Brady Binding Assay

The Brady Binding Assay was developed in our lab to identify the ability of a phage to reversibly bind to a test material (such as a spore or unrelated bacteria) and to remain viable against its original target. In brief, the assay is setup by incubating the phage with the test bacterium, transferring the sample onto a filter, and then rinsing the trapped bacteria to remove un-bound phages. The trapped, rinsed, bacteria are incubated with bacteria of the original phage target and a standard plaque assay is done. Brady binding assays for this study were setup as follows: overnight cultures of *P. larvae* ATCC 9545, *B. laterosporus* field isolate B-2, *Sinorhizobium meliloti* strain B100, and *P. larvae* ATCC 9545 spores were each diluted to 10^4 CFU/ml. The Brady binding assay was setup each time using an MOI of 10^4 with each sample in triplicate. The bacteria were pelleted, supernatant discarded, and the pellets resuspended in 1 ml of phage lysate at a titer of 10^8 pfu/ml, control samples of phage lysate were resuspended in 1 ml of sterile 1/2 × PBHI broth without bacteria, and all samples were incubated for 30 min at room temperature. Each solution was filtered using single-use 0.22 μM vacuum filter to remove all bacteria. The filters were then rinsed with 1 L of 1× phosphate buffered solution (PBS) to release any phages that were not bound to bacteria. The filters were removed, placed in tubes containing 1 ml of 1/2 × PBHI broth, and vortexed for 1 h to dislodge bacteria from the filter. After vortexing, a standard plaque assay was setup using 100 μL of sample (or diluted sample) to incubate 5 or less minutes with 500 μL of overnight *P. larvae* ATCC 9545, plate in 1/2 × PBHI top agar, and incubate overnight. The resulting plaques were counted and data reported as the average ± standard error of the mean. Data was converted into percentages to compare with flow cytometry results by first dividing the average number of plaques from the positive control group in the Brady assay by the percentage of FITC-positive cells in the corresponding positive control group from flow cytometry. The resultant number was used as the denominator to convert the average number of plaques from each group in the Brady assay into a percentage.

Statistics

Data were analyzed using SAS software (SAS Institute Inc., Cary, NC, United States) and the Mixed Procedure method to generate *p*-values, standard deviation, standard error and to determine statistical significance (for **Figures 4**, **8**). For direct count statistics in 3.1, we used Jeffery's 95% confidence interval (Brown et al., 2001) for binomial proportions. For all experiments $\alpha = 0.05$.

RESULTS

Phage PL.Ph-1 Was Specific for *P. larvae* and the *P. larvae* Spores Prepared for Binding Studies Were Pure and Viable

Figure 1 includes an electron micrograph of phage PL.Ph-1 and images of plates demonstrate the lytic nature of the phage used in these studies. PL.Ph-1 is a siphovirus with a prolate head and is the same phage labeled #1 in studies by Brady et al. (2017) which was used in the phage cocktail for hive treatments in that same paper. This phage was selected for further study because, as previously reported, it was the only phage that readily formed plaques on more than 90% of the field isolates to which it was challenged compared to the other 38 phages that formed plaques on an average of only 39.4 ± 2.4% of field isolates (Brady et al., 2017). Challenge of the PL.Ph-1 phage on multiple strains of

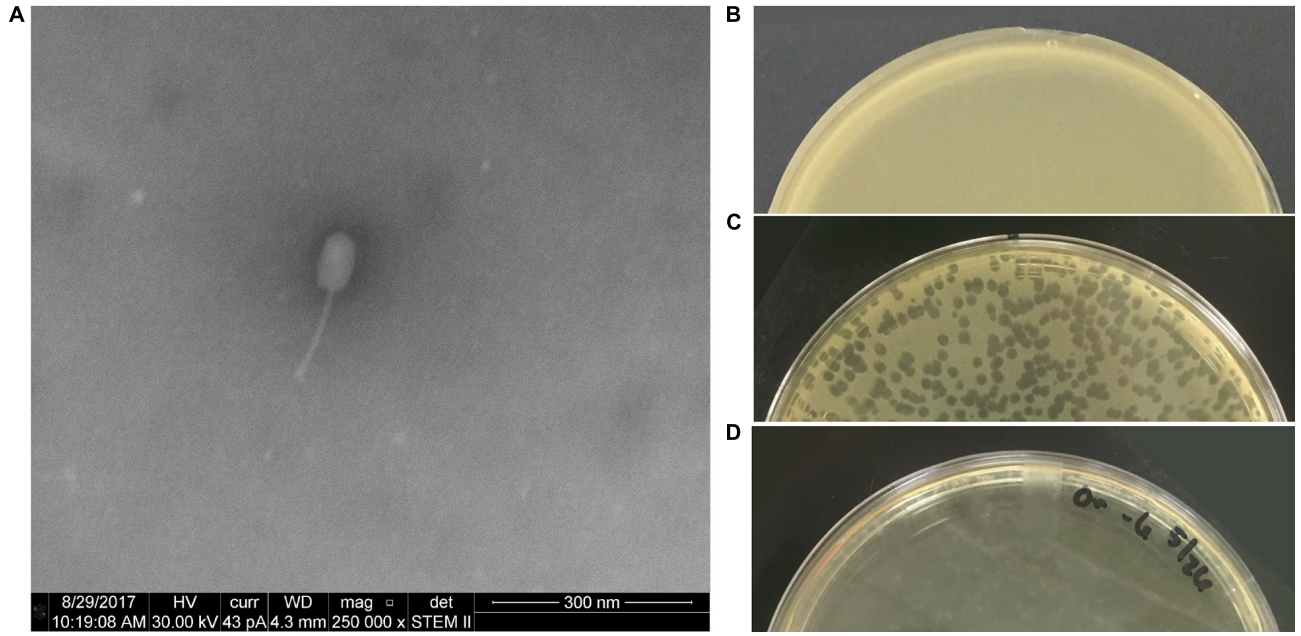

FIGURE 1 | PL.Ph-1 is a siphovirus with a prolate head **(A)** capable of forming plaques on *P. larvae*. A control plate of ATCC 9545 **(B)**, plaques of PL.Ph-1 **(C)**, and a fully lysed plate **(D)** demonstrate the lytic ability of the phage.

B. laterosporus did not yield plaques, which was not surprising since none of the other 38 isolated *P. larvae* phages formed plaques on *B. laterosporus* when tested in our lab.

In order to study phage binding to spores, the *P. larvae* spores to be used for the study were rigorously tested to ensure purity of the prepared spores. A pure spore sample is free of vegetative cells and composed only of spores with few spores-in-mother cells. No vegetative cells were identified in the spore samples using this method and free endospore comprised 92 ± 2.1% of the cells. The samples contained 8.0 ± 2.6% of spores that had not released from their mother cell.

Spore samples were incubated in 1/2 × PBHI broth to further confirm the spore purity of the samples. The purpose of this study was to verify that the spore sample used for subsequent studies did not contain vegetative cells and would not germinate spores in the time span of the experiments. Results of this study are presented in **Figure 2**. Positive controls of vegetative bacteria were incubated with starting concentrations of 10^6, 10^5, and 10^4 CFU/ml. Spores had an approximate concentration of 10^5CFU/ml. The optical density of the spore samples did not change significantly over 17 h in comparison to the vegetative *P. larvae* samples at 10^6, 10^5, and 10^4 CFU/ml over the same amount of time. Spores generated from strain ATCC 9545 do not germinate in 1/2 PBHI liquid media without at least previously being heat activated for 30 min at 70°C (Alvarado et al., 2013); therefore, any increase in optical density of an incubated sample in our studies would result from vegetative bacterial growth in the sample and not from spore germination. These data indicate that the spore samples did not contain any detectable amounts of vegetative bacteria. All studies were conducted with 17 or fewer hours of incubation.

The ethanol-wash treatment used to prepare spores was also tested on vegetative *P. larvae* to verify that the ethanol treatment had killed any surviving vegetative cells in the spore samples. No colonies formed from ethanol wash samples. Spore samples were plated for germination to ensure their viability. After a 48-h incubation, colonies formed on spore-inoculated plates and the colonies were confirmed to be *P. larvae*.

Flow Cytometry Studies Indicated That Phages Bind to Spores and Related Bacteria

Use of flow cytometry to measure phage binding has not previously been reported. We anticipated that phage binding could be quantified using flow cytometry because others have demonstrated that phages could be stained with FITC fluorochrome and then visualized by confocal microscopy (Kelly et al., 2006; Puapermpoonsiri et al., 2010). A flow cytometer takes a reading of each cell in a suspension and reports the fluorescence intensity of each individual cell. Flow cytometry can produce rapid, quantitative results from thousands of individual bacterial cells in each sample while ignoring free-floating phages. We first needed to establish the highest concentration of FITC stain that would not generate background staining on the bacteria. Bacterial samples were incubated with different concentrations of FITC stain to be used for phage staining (**Figure 3**). These results indicated that the bacteria should not be exposed to FITC solutions of 31.3 μg/ml or greater, otherwise background staining of the bacteria would be observed in the flow data and could complicate interpretation in samples containing stained phages. The experiment proceeded with phages in a

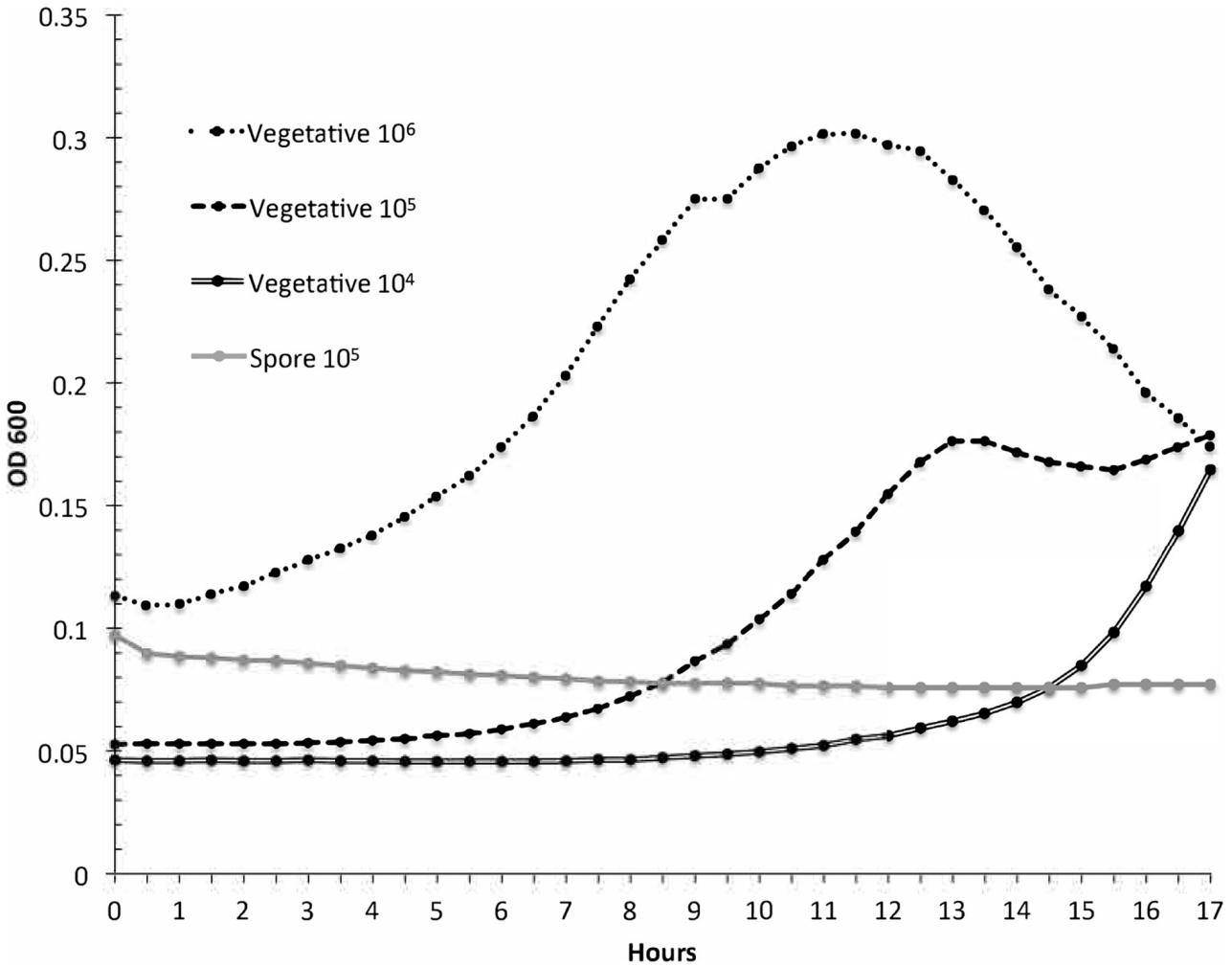

FIGURE 2 | *Paenibacillus larvae* growth curves are used to test spores for contaminant vegetative cells. The optical density was measured for three dilutions of vegetative *P. larvae* and for *P. larvae* spores during a 17-h incubation in broth. The resulting curves indicate that the spore sample did not contain detectable vegetative bacteria.

solution containing 15.6 μg/ml of FITC to be incubated with the test bacteria.

As depicted in **Figure 4**, data analysis of flow cytometry results utilized a series of three plots to identify whether or not the fluorescent phages bound to bacteria. The first two plots were used to select the population to analyze (**Figures 4A,B**) and the third plot reported the number of cells with each intensity of FITC staining (**Figure 4C** is a negative sample and **Figure 4D** is a positive sample). On the histogram of the FITC reading, cells that are positive indicate bacteria with phages attached.

FITC-stained phages were mixed with vegetative *P. larvae*, *B. laterosporus*, *S. meliloti*, and spores of *P. larvae*. The average percentage of FITC-positive bacterial cells for each is reported in **Figure 5**. *P. larvae*, *B. laterosporus*, and *P. larvae* spores treated with labeled phages are significantly different ($p < 0.0001$) from untreated samples where *S. meliloti* treated with phages did not have a statistical difference from an untreated sample ($p = 0.3725$). **Table 2** contains the *p*-values for statistical analysis of data from the last four columns in **Figure 5** to compare bacterial sample containing phages with each other. Phage binding to *S. meliloti* and to *B. laterosporus* is significantly different compared to phage binding to *P. larvae*, regardless of whether it is vegetative or spore forms of *P. larvae*. Phage binding to *S. meliloti* is not statistically different from phage binding to *B. laterosporus*. Phage binding is also not statistically different when comparing vegetative and spore forms of *P. larvae* to each other. These data support the hypothesis that *P. larvae* phages bind to spores.

Electron Microscopy Visually Confirmed That Phages Attach to Spores

Electron microscopy was used to visualize phage-binding to spores. **Figure 6** includes electron micrographs of vegetative *P. larvae* incubated with phages. Phages were observed in

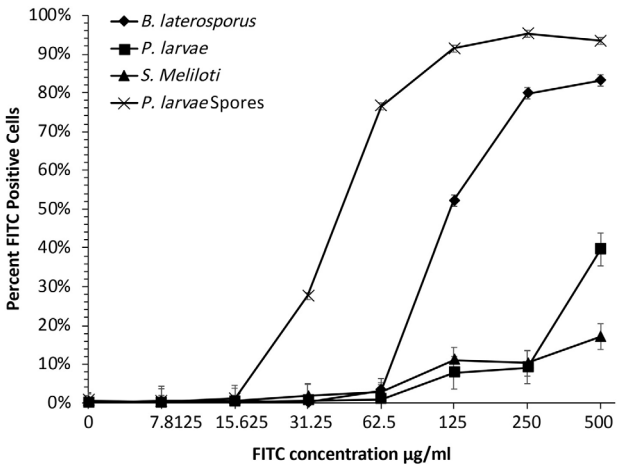

FIGURE 3 | For detection of FITC-stained phages, FITC must be optimized to a concentration below where bacteria will absorb detectable stain. Bacterial samples were dosed with seven FITC concentrations to determine background-fluorescence of bacteria. Samples were compared to untreated groups to establish what concentration of FITC caused background staining. Bacterial fluorescence was detected at doses of 15.625 µg/ml or higher.

horizontal and vertical positions with respect to the bacterium. Most commonly, the phages were attached by the end of the tail fiber to the side of the vegetative bacterium. Visible damage occurred to the bacterial wall upon infection, and late-stage infection was observed as an eruption from the bacterial wall.

Figure 7 includes electron micrographs of *P. larvae* spores incubated with phages. Phages were observed in horizontal and vertical positions on the spores. Spores that had not yet released from the mother cell were equally prone to have phages attached, and the phages appeared equally able to attach to the spore side as to the mother cell side (**Figure 6A**). Phages were observed in both horizontal and vertical positions on spores. Some phages clearly attached to the spore by the end of the tail fiber (**Figure 6B**). Phages appeared to be attached in higher abundance on vegetative cells than on spores as observed in electron micrographs. Our imaging methods did not distinguish between empty or full capsids, so no interpretation can be made regarding whether or not a phage injected its DNA into a spore or not.

Results of the Binding Assay Demonstrated That Phages Reversibly Bind to Spores and That the Phages Are Still Infectious

The Brady Binding Assay includes a short incubation period with phages and a long rinse of the bacteria prior to a plaque assay (**Figure 8**). Since the thoroughly rinsed bacteria are used as the source of phages for the plaque assay, plaques indicate that binding to the challenge bacteria occurred during the initial short incubation period. Phages can produce plaques in two ways: (1) the phages are able to productively infect challenge bacteria during the first step and then produce plaques on their intended host in the plaque assay, or (2) the phages can exhibit reversible binding wherein phages bind to the challenge bacteria in the first step and release to infect and produce plaques on their intended host in the plaque assay.

Results of the Brady assay are presented in **Figure 9** and **Table 3**. The "phages only" control indicates the resultant number of phages that become mechanically trapped on the filter during the Brady assay. A statistically significant number of plaques formed from all test bacteria samples compared to the phage only samples. By comparison, *P. larvae* spores formed plaques significantly higher than the unrelated *S. meliloti* and statistically lower than vegetative *P. larvae*. The number of plaques from *B. laterosporus* was surprisingly not statistically different from that of vegetative *P. larvae*, nor was it statistically different from that of *P. larvae* spores ($\alpha = 0.01$). These results indicate that *P. larvae* phages bind with significant abundance to vegetative *P. larvae, P. larvae* spores and to vegetative *B. laterosporus*.

Comparative Results of Flow Cytometry Detection and the Brady Binding Assay Allow Interpretation of the Phage-Binding Ability of PL.Ph-1

Results from the Brady assay were converted into percentages so that the data could be compared between the two quantitative methods used in our research. **Table 4** provides a comparison between flow cytometry data and Brady assay results and summarizes a proposed interpretation of these data.

DISCUSSION

The body of evidence from our data supports that *P. larvae* phages do, indeed, bind to *P. larvae* spores. Differences in results from flow cytometry and from the Brady binding assay data should come under careful scrutiny considering that, while still supportive of our hypothesis, these two detection methods yielded different results. As presented in **Table 4**, we believe that these data evidence important information about what each methods is able to detect, as well as the reversibility, and potentially the evolutionary survival strategies of the phages.

A few factors should be considered when interpreting flow cytometry data. The phages were stained with FITC for detection of phage binding to bacteria. The more phages that attach to an individual bacterium, the brighter the fluorescence of that bacterium. However, we did not report the brightness of the cell, but rather the total number of cells that are above a threshold of brightness. This means that the cutoff point, as indicated in **Figures 4C,D**, will count cells as positive, as long as enough phages are attached to fluoresce the bacteria above the cutoff. Based on the fact that more plaques were formed in the Brady assay for *B. laterosporus* than were detected by flow cytometry, our cutoff point for flow

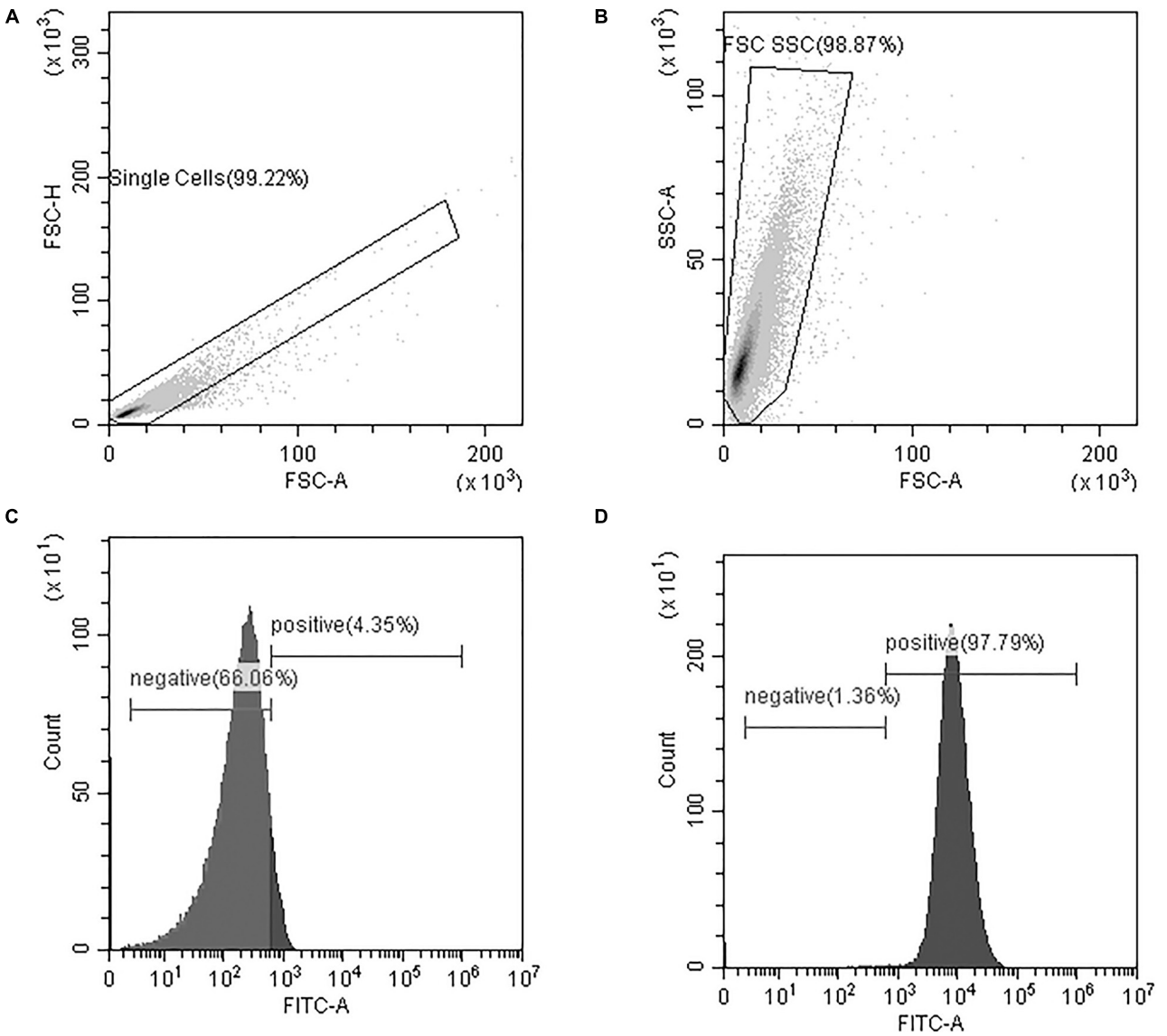

FIGURE 4 | Flow cytometry data analysis included **(A)** a gate to isolate single cells from the sample based on a plot of Forward Scatter Area and Height readings (FSC-A and FSC-H, respectively) **(B)** a gate to eliminate small and large debris based on Forward and Side Scatter Area readings (FSC-A and SSC-A, respectively), and **(C,D)** a histogram of the gated cells with FITC fluorescence-intensity on the x-axis and the number of cells on the y-axis. The FITC histograms yield peaks in the positive or negative regions of the plot and percentages are reported. Representative samples are shown for **(C)** a FITC-negative sample, and **(D)** a FITC-positive sample. At least 50,000 cells were analyzed from each sample.

cytometry excludes bacteria that may have a very low number of phages per bacterium.

Initially we posited that, since spores are inactive, phages would not permanently bind to spores, but could reversibly bind in order to release from the spore and infect vegetative bacteria. Since the Brady assay does not support activation of spores, plaques produced from the spore sample must indicate reversible binding and must further indicate that the phages are still active for infection after binding and releasing from the spores. The results are supportive of this interpretation because spore and vegetative samples were not different by flow cytometry, but were, statistically, slightly lower in the spore sample by Brady assay. The statistically lower number of plaques in the spore sample by Brady assay likely represent spores that retain the bound phages. Some phages are expected to remain on spores for better survival of the phages. Since spore germination in a bee infection only occurs inside the larval gut, phages that are attached to spores must have survival and transmission strategies to eventually gain access to vegetative bacteria of their host.

The plaque assay used as part of the Brady assay technique generates a single plaque regardless of whether the bacterium in that location carried a high or low number of phages. This assay, therefore, provides a good view of what percentage of cells have

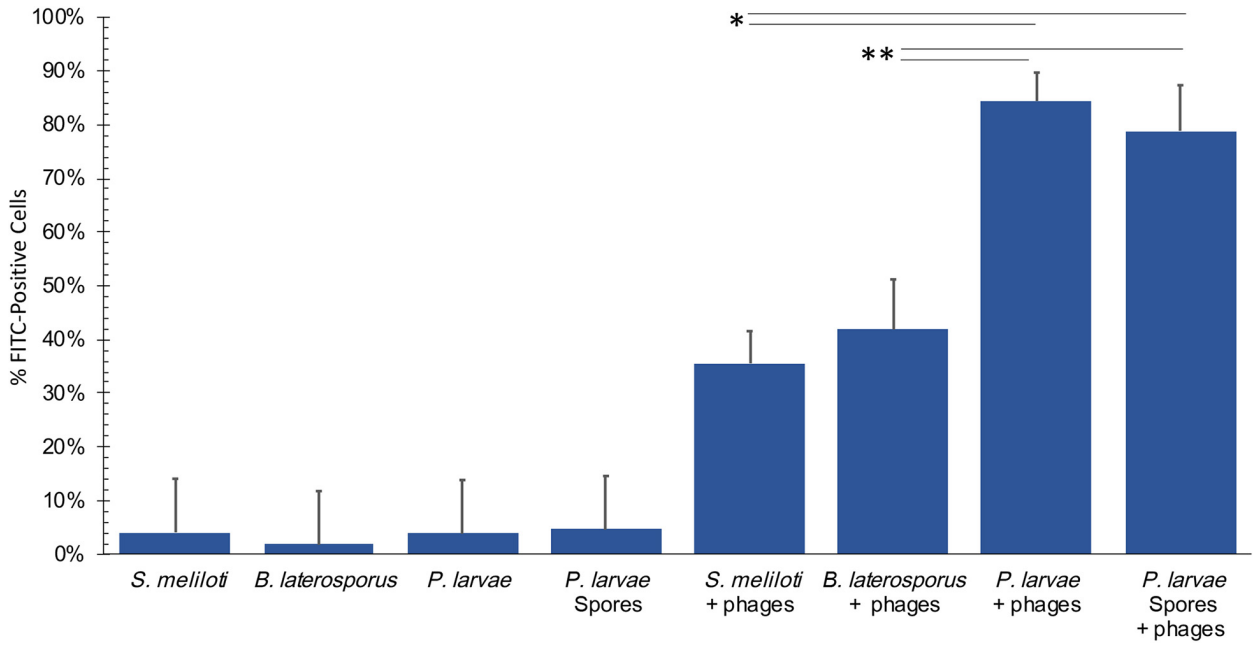

FIGURE 5 | Flow cytometry results detect phages bound to bacteria and spores. Negative controls (first four columns), were low, and all samples with phages were statistically significant compared to the negative controls excluding *S. meliloti*. Statistically significant data is indicated with asterisks. See **Table 2** for averages and *p*-values from the statistical analysis of samples with phages.

TABLE 2 | Average percentage of FITC-positive cells by flow ctyometry.

	Average % of FITC-positive cells	***S. meliloti***	***B. laterosporus***	***P. larvae***	***P. larvae* spores**
S. meliloti	29.1 ± 5.7%	–	0.8127	0.0001	0.0001
B. laterosporus	41.8 ± 6.5%		–	0.0004	0.0056
P. larvae	84.4 ± 5.7%			–	0.9979
P. larvae spores	78.8 ± 6.5%				–

P-values are from statistical results comparing bacterial sample challenged with phages.

phages attached, but does not represent how many phages were attached to a single bacterium. Furthermore, if the phage can infect the test bacterium, as is the case with vegetative *P. larvae*, then the Brady assay results cannot reflect whether phage binding is reversible since the phages can productively infect and form a plaque without having to reversibly bind. Data in **Table 3** confirms that the results of the Brady assay directly reflect that of Flow cytometry for vegetative *P. larvae*.

For our studies, we intentionally did not heat activate spores so that the spores would not be able to germinate during an assay. Non-germinating spores would ensure that any plaques that formed on the vegetative lawn in a Brady assay would be from a surface release of the phages from the spores rather than an infection of the spores themselves. In like manner, our study used phages that cannot productively infect *B. laterosporus* or *S. meliloti*, so that any plaques formed on the *P. larvae* vegetative lawn would again be a result of binding and subsequent surface release of the phages from these bacteria rather than an infection of them. Non-binding phages were rinsed away prior to the challenge bacteria being placed with the vegetative *P. larvae*. We conclude that the observed plaques must come from reversible binding.

Results from *B. laterosporus* indicate that *P. larvae* phages bind to this non-host bacterium, but can readily reverse binding and productively infect their *P. larvae* host. The high number of plaques observed from this sample in contrast to the low flow cytometry counts, indicates that many *B. laterosporus* bacteria have a small number of phages attached that can reversibly bind and subsequently infect *P. larvae*. For instance, a single phage on *B. laterosporus* would be very unlikely to be detected by flow cytometry, but would yield a plaque in the Brady assay. Reversible binding may be a mechanism of survival of the phages but does not preclude other mechanism, such as capture and protection of phage DNA within bacterial spores of the host bacterium (Silver-Mysliwiec and Bramucci, 1990; Walter, 2003; Sonenshein, 2016; Gabiatti et al., 2018). The increased binding and reversible capability of *P. larvae* phages on a different bacterial species than the phages' host and which bacterial species resides in the same niche space as the phages' host (in this case *B. laterosporus*) is a newly identified mechanism for phage survival.

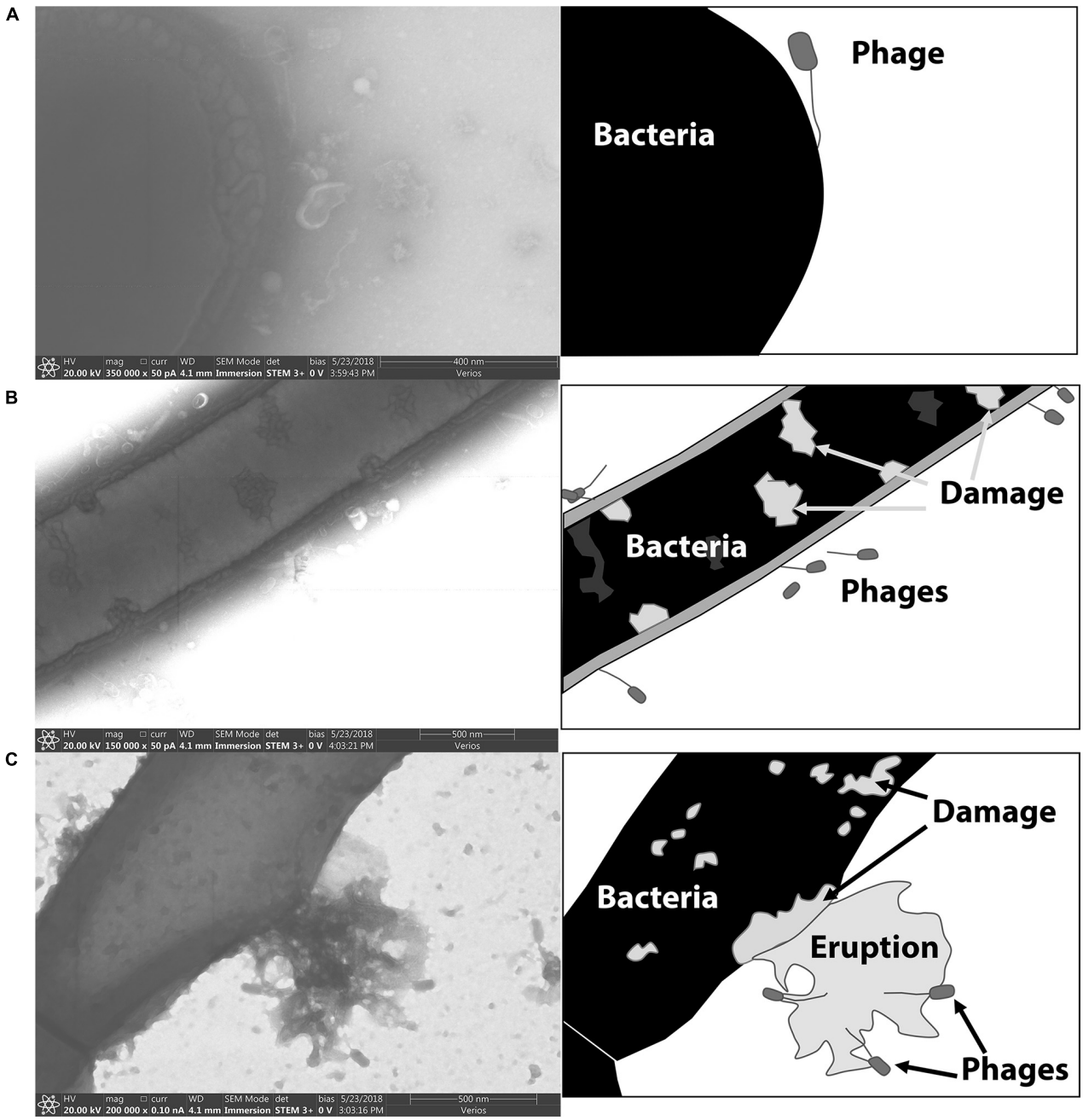

FIGURE 6 | STEM images of phages bound to the vegetative form of *P. larvae*. Cartoon depictions in the panels to the right of each electron micrograph include labels of notable items. **(A)** A phage is attached to the end of a vegetative *P. larvae* bacterium. **(B)** Several phages are visibly attached to the sides of vegetative *P. larvae*. **(C)** A vegetative *P. larvae* bacterium is undergoing lysis after productive phage infection.

Brevibacillus laterosporus is a firmicutes bacterium distinctly separate from *P. larvae* but recently identified as having significant genetic similarities (Berg et al., 2018) in addition to their both residing in beehives. We did not anticipate the high numbers of plaques generated from *B. laterosporus* bacteria in the Brady assay because *P. larvae* phages used for this study do not productively infect *B. laterosporus*. *B. laterosporus* has been debated as to whether it is a commensal or a pathogenic bacterium in honeybees (Charles and Nielsen-LeRoux, 2000; Ruiu et al., 2012, 2014; Ruiu, 2013; Bashir et al., 2016; Marche et al., 2016; Mura and Ruiu, 2017). Studies indicate that *B. laterosporus* at least increases in prevalence during an *P. larvae* infection in the hive (Alippi et al., 2002). The presence of *B. laterosporus* during AFB infections led to the development of a bystander phage therapy for AFB (Brady et al., 2018), albeit the phage for bystander treatment was a *B. laterosporus*

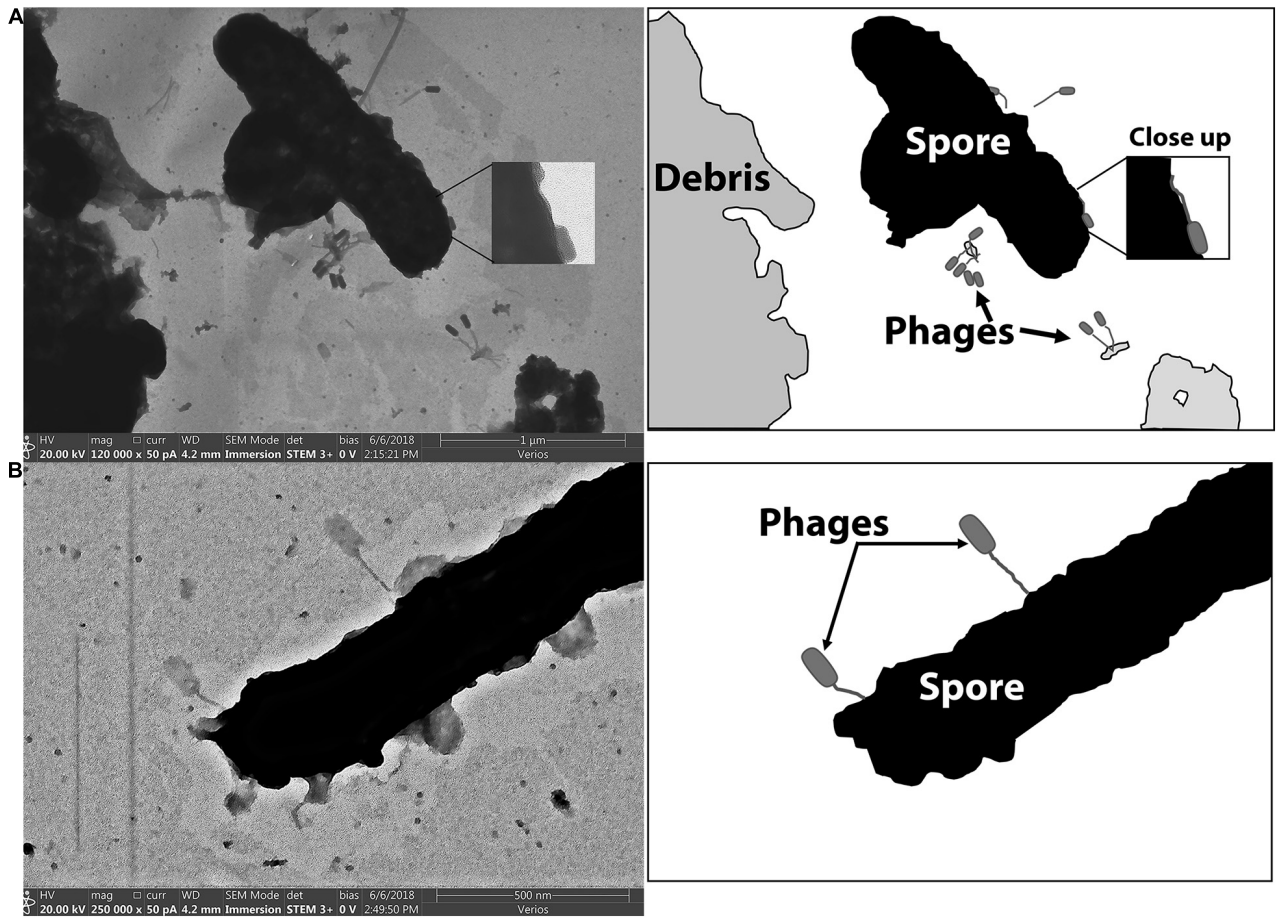

FIGURE 7 | STEM images of phages bound to the spore forms of *P. larvae*. Cartoon depictions in the panels to the right of each electron micrograph include labels of notable items. **(A)** Phages are attached to a mother-in-spore of *P. larvae*. **(B)** Phages are attached to a fully-formed spore of *P. larvae*.

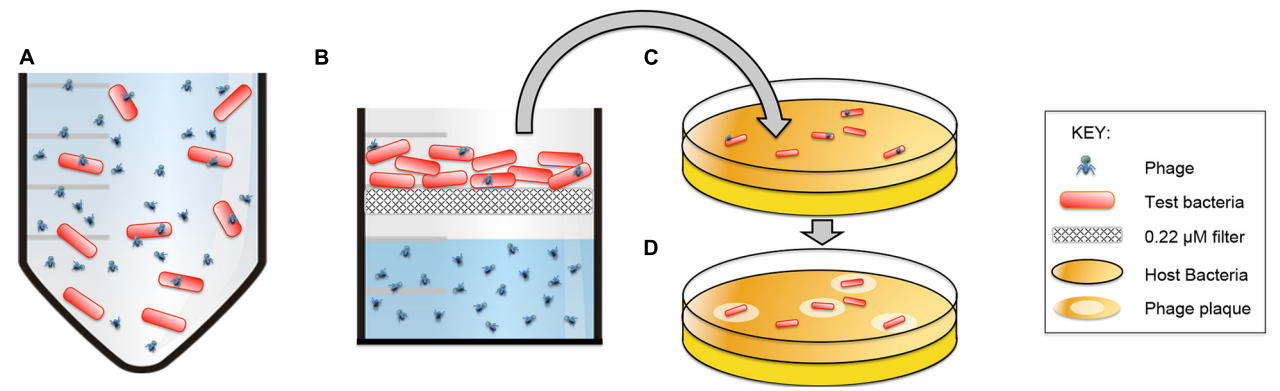

FIGURE 8 | The Brady Binding Assay detects viable phages that directly infect and/or reversibly-bind to bacteria. **(A)** Phages are incubated with test bacteria. **(B)** Bacteria are caught on a filter and heavily rinsed with PBS to remove non-bound phages. **(C)** Bacteria are removed from the filter and plated with their host bacteria to incubate for 24 h. **(D)** Viable phages form visible plaques on the host bacteria which indicates that direct infection or reversible binding has occurred between the phages and the test bacteria.

phage. Since the *P. larvae* lifecycle includes spore transmission, we anticipated that phages must have a mechanism of survival between the spore stage and the next germination. While we hypothesized that this survival would be due to spore binding, we did not anticipate an alternative hypothesis that *P. larvae* phages bind to *B. laterosporus* in a survival strategy, as our data

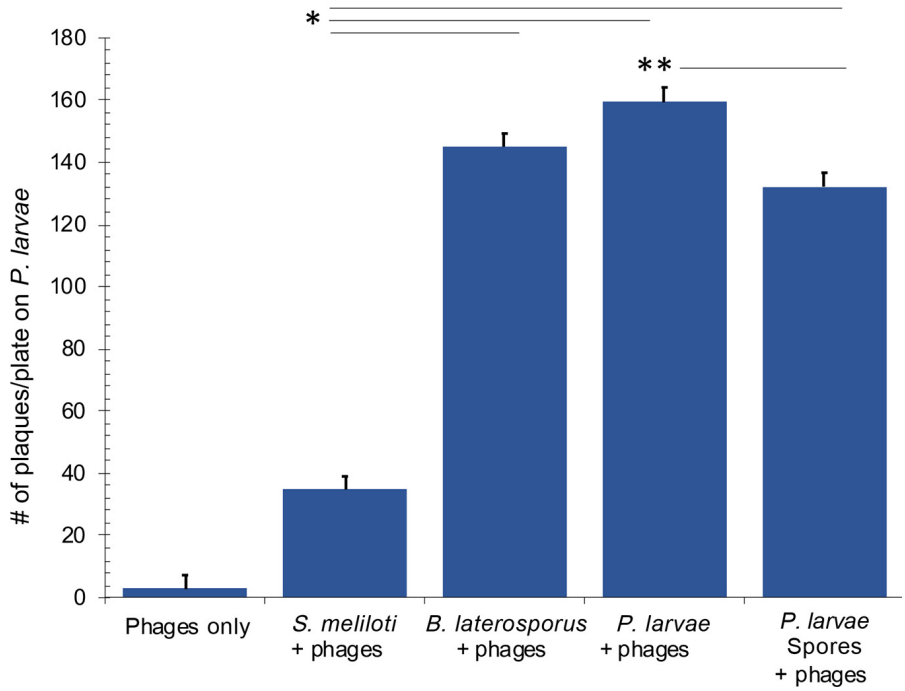

FIGURE 9 | Results of the Brady assay indicate that phages bind to vegetative and spore samples of *P. larvae*, and to vegetative *B. laterosporus*. All samples where phages were challenged with bacteria were statistically different from the phages only control. Statistically significant data between test bacteria samples are indicated with asterisks. See **Table 3** for averages and *p*-values from the statistical analysis.

TABLE 3 | Average plaque counts from the Brady Binding Assay.

	Average plaque counts	*S. meliloti*	*B. laterosporus*	*P. larvae*	*P. larvae* spores
Phages only	3 ± 10	<0.0001	<0.0001	<0.0001	<0.0001
S. meliloti	35 ± 9	–	<0.0001	<0.0001	<0.0001
B. laterosporus	145 ± 9		–	0.1925	0.2494
P. larvae	159 ± 10			–	0.0018
P. larvae spores	132 ± 9				–

P-values are from statistical analysis comparing phage binding in each bacterial sample challenged with phages.

TABLE 4 | Comparison of phage binding capability data from results of Flow cytometry and the Brady Binding Assay.

	% binding by Flow cytometry	% binding by Brady assay	Phage-binding ability	Fluorescence intensity for Flow cytometry detection	Percentage of cells with phages attached	Reversibility of binding
S. meliloti	29.1 ± 5.7%	18.4 ± 2.2%	Very low number of phages per bacterium	Dim to undetected	Low percentage of cells have phages	Low reversibility?
B. laterosporus	41.8 ± 6.5%	76.8 ± 2.2%	Low number of phages per bacterium	Dim to undetected	High percentage of cells have phages	High reversibility
P. larvae	84.4 ± 5.7%	84.4 ± 2.5%	Many phages per bacterium	Bright	High percentage of cells have phages	Moderate reversibility?
P. larvae spores	78.8 ± 6.5%	70.0 ± 2.4%	Moderate number of phages per bacterium	Bright	High percentage of cells have phages	Moderate reversibility

indicate. While *B. laterosporus* is able to sporulate, we have not yet explored whether or not *P. larvae* phages can also bind to *B. laterosporus* spores. Studies with *B. laterosporus* spores as well as other bacterial species will be interesting to explore for the level of phage binding. Since *B. laterosporus* is also found in beehives and is even more often found alongside *P. larvae*, we posit that this phage binding is an evolutionary survival strategy of the phages.

Electron microscopy images visually demonstrated that these phages bind to the surface of spores in various orientations.

Other researchers used cryotomography of T4 phages to reveal different phage orientations during the infection process (Leiman et al., 2010; Hu et al., 2015; Taylor et al., 2016). Their results suggest that long tail fibers first bind to target bacteria and generate pressure that triggers the release of short tail fibers from the baseplate. The short tail fibers from the baseplate bind to specific receptors on the surface of the bacterium, which in turn erects the phage and triggers the injection of DNA into the cell (Leiman et al., 2010; Vinga et al., 2012; Hu et al., 2015; Taylor et al., 2016). Both horizontal and vertical binding was apparent in our electron microscopy samples of both vegetative and spore *P. larvae*. If the erect phages have bound and injected DNA into the cell, then *P. larvae* phages may be able to directly infect *P. larvae* spores similar to other spore-infecting phages (Fu et al., 2011). The alternative phage orientation on the bacterium and spores in our images may indicate differences between reversible and irreversible binding, and/or may indicate that DNA injection occurs with both the vegetative and spore forms of *P. larvae*. Our staining and imaging methods do not allow distinction of whether or not DNA was injected.

In our samples, approximately 8% of spores had not exited the mother cell. We anticipated that vegetative cell receptors would remain on the mother cell side to which phages could bind. By electron microscopy, phages were observed attached to both the spore side and the mother side of these spores. The quantitative data well exceeded that of 8% of phage-positive spores using flow cytometry and by the Brady assay. Further, electron microscopy clearly captured phages attached to mature spores, indicating that phage binding can occur on a fully formed *P. larvae* spore. Since the phage-bound spores were bright by flow cytometry, we anticipate that a moderate to high number of phages attached to each spore, and the majority of spores detached at least one phage in order to form plaques to result in such a high number of plaques from these samples. Whether or not the phages that remained on the spores had already or could infect a spore to yield a productive infection upon germination is yet to be determined.

Our results directly support the hypothesis that spore-binding phages may prevent reinfection from spores of a recovered beehive. In field studies of AFB-infected hives treated with *P. larvae* phages, not only did beehives recover from AFB in less than 2 weeks but the hives also did not become re-infected for the following 6 months of observation (Brady et al., 2017). This contrasts with the fact that bystander phage therapy, which uses phage-induced toxins capable of killing vegetative *P. larvae*, can cure active AFB within 2 weeks but AFB re-emerges within 1 month of treatment (Brady et al., 2018), and furthermore that antibiotic treatment of hives also experience re-emergence of AFB within months of clearing an active infection using antibiotics (Alippi, 1996; Alippi et al., 1999; Brady et al., 2017). These studies indicate that if a hive is treated to kill only vegetative bacteria and spores are not neutralized, then a re-infection will occur. Evidence of spore binding as presented in this report provides a mechanistic explanation for the success of the phages used in the hive treatment studies. Reversible binding of *P. larvae* phages on spores and on *B. laterosporus* increases the likelihood of the phages encountering vegetative *P. larvae* when spores germinate or when *B. laterosporus* expands as a secondary infection to AFB. *B. laterosporus* may be a commensal or a pathogenic bacteria in honeybees (Alippi et al., 2002). As a commensal, reversible binding to this bacteria is a viable mechanism for retaining phages within the hive for protection against reinfection by *P. larvae*. Alternatively, phages that can attach to *P. larvae* spores may directly infect or may release from and infect newly germinated vegetative *P. larvae*.

Although this work did not show that the phages directly kill spores, other phages have been identified that do (Walter, 2003; Fu et al., 2011). By hunting for phages that specifically bind to and/or destroy spores, phage cocktail therapies against sporulating bacterial species will likely have a greater potential for functionality with a possibility of preventing recurrent infections caused by spores. The results presented in this report are the first to demonstrate that *P. larvae* phages can be bind to both *P. larvae* spores and to vegetative *B. laterosporus*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

TB, CF, and SH: conceptualization. TB, JW, CF, and CR: lab work. TB, JW, CR, DE, and SH: analysis. TB: writing – original draft preparation. CR, DB, and SH: writing – review and editing. DB and SH: supervision. SH: project administration. TB and SH: funding acquisition. All authors contributed to the article and approved the submitted version.

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REVIEW
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A Phage Therapy Guide for Clinicians and Basic Scientists: Background and Highlighting Applications for Developing Countries

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Approximately 10% of global health research is devoted to 90% of global disease burden (the so-called "10/90 Gap") and it often neglects those diseases most prevalent in low-income countries. Antibiotic resistant bacterial infections are known to impact on healthcare, food security, and socio-economic fabric in the developing countries. With a global antibiotic resistance crisis currently reaching a critical level, the unmet needs in the developing countries are even more striking. The failure of traditional antimicrobials has led to renewed interest in century-old bacteriophage (phage) therapy in response to the urgent need to develop alternative therapies to treat infections. Phage therapy may have particular value in developing countries where relevant phages can be sourced and processed locally and efficiently, breaking specifically the economic barrier of access to expensive medicine. Hence this makes phage therapy an attractive and feasible option. In this review, we draw our respective clinical experience as well as phage therapy research and clinical trial, and discuss the ways in which phage therapy might reduce the burden of some of the most important bacterial infections in developing countries.

Keywords: bacteriophage, developing countries, antibiotic resistance, mortality, disease burden

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INTRODUCTION

In 1990, the Global Burden of Disease Study (GBD) began to monitor the burden of specific health conditions in populations at national, regional and global levels in order to inform health policies especially in developing countries (Michaud, 2009). Almost two decades on, the 2017 GBD report indicated an improvement in the overall mortality from communicable infections but pointed to the continuing heavy socio-economic and public health burdens in developing countries (GBD Causes of Death Collaborators, 2018; **Figure 1**).

Infectious diseases disproportionately affect developing countries which, when combined with malnutrition, unhealthy living conditions and unsafe drinking water, drive morbidity and mortality and economic injury. The global expansion of antibiotic resistance (AMR) not only exacerbates this but also threatens to reverse the reductions in mortality and morbidity from endemic infections that are enjoyed in developing countries (**Figure 1**). A recent report estimated that AMR will contribute

an excess of 10 million deaths and a GDP loss of $100 trillion USD by 2,050 if effective measures are not taken to contain it (O'Neill, 2016).

Developing countries have in common limited healthcare systems and fragile economies and are ill-equipped to manage a growing infectious diseases burden despite all efforts from international health and humanitarian organizations (Bhutta et al., 2014).

In 2014, the first World Health Organisation (WHO) global surveillance report on antibiotic resistance showed that > 50% of clinically important bacteria from five of the six WHO regions have resistance against third generation cephalosporins, fluoroquinolones and carbapenems, and attributed 45% of deaths in Africa and South East Asia to multi-drug resistant (MDR) bacterial infections (WHO, 2014). Poor socioeconomic conditions, illiteracy, limited healthcare facilities, and unregulated antimicrobial use in humans and animals are important contributors to undesirable antibiotic resistance trends and their consequences (Aarestrup, 2012; Ayukekbong et al., 2017).

In recognition of this, a consortium of major pharmaceutical companies is creating a $1 billion for-profit venture in support of small biotechnology companies developing mid-stage antibiotics (Silverman, 2020). While a comprehensive and integrated collaboration to antibacterial compounds and vaccines at global level is currently underway (Tong, 2020), the pathway to market access remains a barrier. The golden era of antibiotics continues to fade and there is an urgent need to develop and implement novel therapeutic strategies for infectious (Alanis, 2005).

The century-old science of bacteriophage (phage) therapy was largely neglected after the advent of antibiotics (Summers, 2001) but there remain distinct advantages. Phages are highly specific antibacterial agents that cause much less collateral damage to the microflora than conventional antibiotics that can be applied directly to human tissues without causing harm (d'Herelle, 1931; Weber-Dabrowska et al., 1987; Petrovic Fabijan et al., 2020a) and their abundance means they can be locally sourced, processed and packaged (Nagel et al., 2016).

Good Manufacturing Practice (GMP) preparations free of bacterial contaminations (especially lipopolysaccharides) for intravenous (IV) administration are a manufacturing challenge (not just in developing countries) but alternative administration of phages topically and orally (**Figure 2**) can be effective and feasible (Gill and Hyman, 2010). Here, we discuss some prominent infections for which phage therapy might be considered (**Table 1**).

Typhoid

Typhoid fever is an occasionally fatal systemic infection caused by *Salmonella typhi* and *paratyphi* strains, responsible for 11–20 million cases and 128,000–161,000 deaths globally each year (Ochiai et al., 2008; Mogasale et al., 2014). A new typhoid conjugate vaccine with longer immunity and better safety profile in children <2 years age has recently been approved (Shakya et al., 2019) but currently available vaccines do not provide long-lasting immunity and vaccination has not been widely implemented in endemic countries.

Extensively drug resistant (XDR) *Salmonella typhi* (to first line antibiotics ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole, as well as fluoroquinolones and third generation cephalosporins) is now widespread in countries like Pakistan (Klemm et al., 2018) and is regularly imported to the United States (Chatham-Stephens et al., 2019), Australia (Howard-Jones et al., 2019), Canada (Wong et al., 2019), the United Kingdom (Klemm et al., 2018), and other countries in Europe (Kleine et al., 2017; Engsbro et al., 2019; Lopez-Segura et al., 2019; Procaccianti et al., 2020). An extensively drug resistant *Salmonella typhi* of a different haplotype (H58) from the epidemic strain in Pakistan (H55) has also emerged in Africa (Akinyemi et al., 2015; Phoba et al., 2017) which is responsive only to last-line hospital intravenous carbapenem antibiotics.

Physicians have used phages to treat typhoid for nearly a century (Smith, 1924) and historical anecdotes includes both oral and intravenous therapy (d'Herelle, 1931). Phages were used successfully in major outbreaks in Los Angeles from 1936 to 1949 (Knouf et al., 1946) and Quebec from 1946 to 1949 (Desranleau, 1948, 1949) and phage therapy for typhoid is once more in focus as antibiotics are failing. The widespread incidence of typhoid fever in low-income countries suggests that their natural predator phages should be present in the environment as well but international biobanks are already acting as vital repositories. Phages with lytic activity against an XDR *Salmonella typhi* strain isolated in the Democratic Republic of the Congo (Kakabadze et al., 2018) were identified from the phage library at the Eliava Phage Therapy Center in Georgia, where a Phage Biobank of obligately lytic phages against most common human pathogens is kept. Typhoid fever may be an ideal candidate for "re-introduction" of phage therapy.

Cholera

Cholera is a self-limiting, rapidly dehydrating secretory diarrheal disease of humans caused by toxigenic strains of the Gram-negative bacterium *Vibrio cholerae*. It is a major cause of mortality and morbidity in developing countries of Asia and Africa and is associated with poor sanitation and lack of clean drinking water (WHO, 2017a; **Figure 3**), with outbreaks often following war or natural disaster (Gupta et al., 2016). In 2018, nearly half a million cases and 3,000 deaths were reported (WHO, 2018). However, lack of diagnostic facilities, inadequate disease surveillance and fear of adverse effects on trade and tourism may all contribute to significant underreporting and WHO estimated the real case load to be nearly 3 million annually in endemic areas with 95,000 deaths (WHO, 2017a), more than half of these being in children ≤ 5 years old (Ali et al., 2015).

Currently, there are three killed whole-cell oral vaccines prequalified by WHO for use in children > 1 year and adults (WHO, 2017a; Seo et al., 2020) and a global stockpile has been created. Millions of doses have been administered and the WHO Global Task Force on Cholera Control aims to end cholera by 2030 through improved surveillance, vaccination, and implementation of improved water, sanitation, and hygiene in "hotspot" areas to reduce incidence and transmission (Zaman et al., 2020). Despite this, cholera has recently

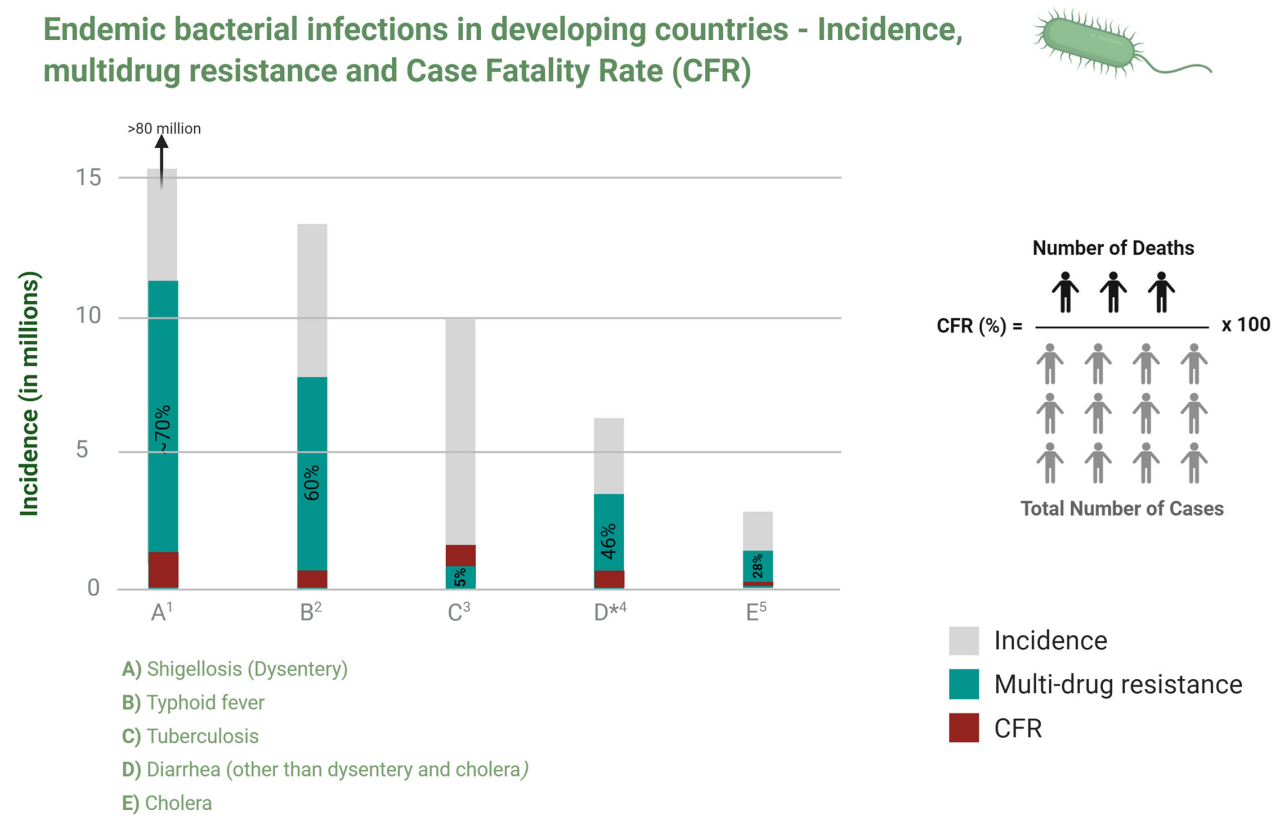

FIGURE 1 | Incidence, multidrug resistance and case fatality ratio of common bacterial infections in developing countries. Data is taken from studies cited in relevant disease sections in the text.

returned to the Americas with ongoing transmission in Haiti (Ganesan et al., 2020; **Figure 3**).

Management of cholera requires aggressive fluid and electrolyte replacement, but antibiotic treatment may decrease diarrhea by 50% and reduce shedding of viable organisms by days (Harris et al., 2012). Chemoprophylaxis within households may be effective but is not recommended by WHO because of the risk from AMR. Most *V. cholerae* in endemic areas are now resistant to the commonly used antibiotics (Dengo-Baloi et al., 2017; Rijal et al., 2019; Verma et al., 2019; Chatterjee et al., 2020).

An inverse relationship between the presence of virulent cholera phages and *V. cholerae* in environmental water samples coincides with the seasonality of disease occurrence in surrounding populations. This indicates a key role for phages in cholera epidemiology (Faruque et al., 2005) and makes biological control using phages an attractive option. Phages have been used to treat cholera since d'Herelle first linked the decline in mortality during a cholera epidemic in India in the 1920s to phages in stool and began to treat patients with oral vibriophages. He achieved a dramatic reduction in mortality with early use of oral phages (d'Herelle, 1929) and subsequent field trials achieved remarkable results in controlling localized epidemics (Summers, 1993). Several later trials reported successful prophylaxis (Sayamov, 1963) but relatively little effect on duration of established diarrheal illness (Monsur et al., 1970) or pathogen excretion (Marcuk et al., 1971).

Therapeutic effectiveness of cholera phages has also been shown in more controlled experiments using animal models, even with single phages (Bhandare et al., 2019). A "cocktail" of five specific phages given 6 and 12 h before *V. cholerae* challenge in adult rabbits slightly reduced both disease severity and bacterial load (Jaiswal et al., 2013) and another study using three phages in combination up to 24 h before *V. cholerae* challenge prevented infection in infant mouse and rabbit models, without emergence of resistance (Yen et al., 2017). It may be that poor choice of phages and/or trial design have contributed to previous failures and it seems clear that rigorous clinical trials of well-selected vibriophages are warranted.

Shigellosis (Bacillary Dysentery)

Shigellosis is a major public health problem in low-middle income countries and an important cause of morbidity in industrialized countries (Kotloff et al., 1999). It is generally a self-limiting diarrheal illness of up to 10 days but severe cramps and mucosal bleeding are not uncommon. Complications include sepsis, encephalopathy, hemolytic uremic syndrome and, rarely,

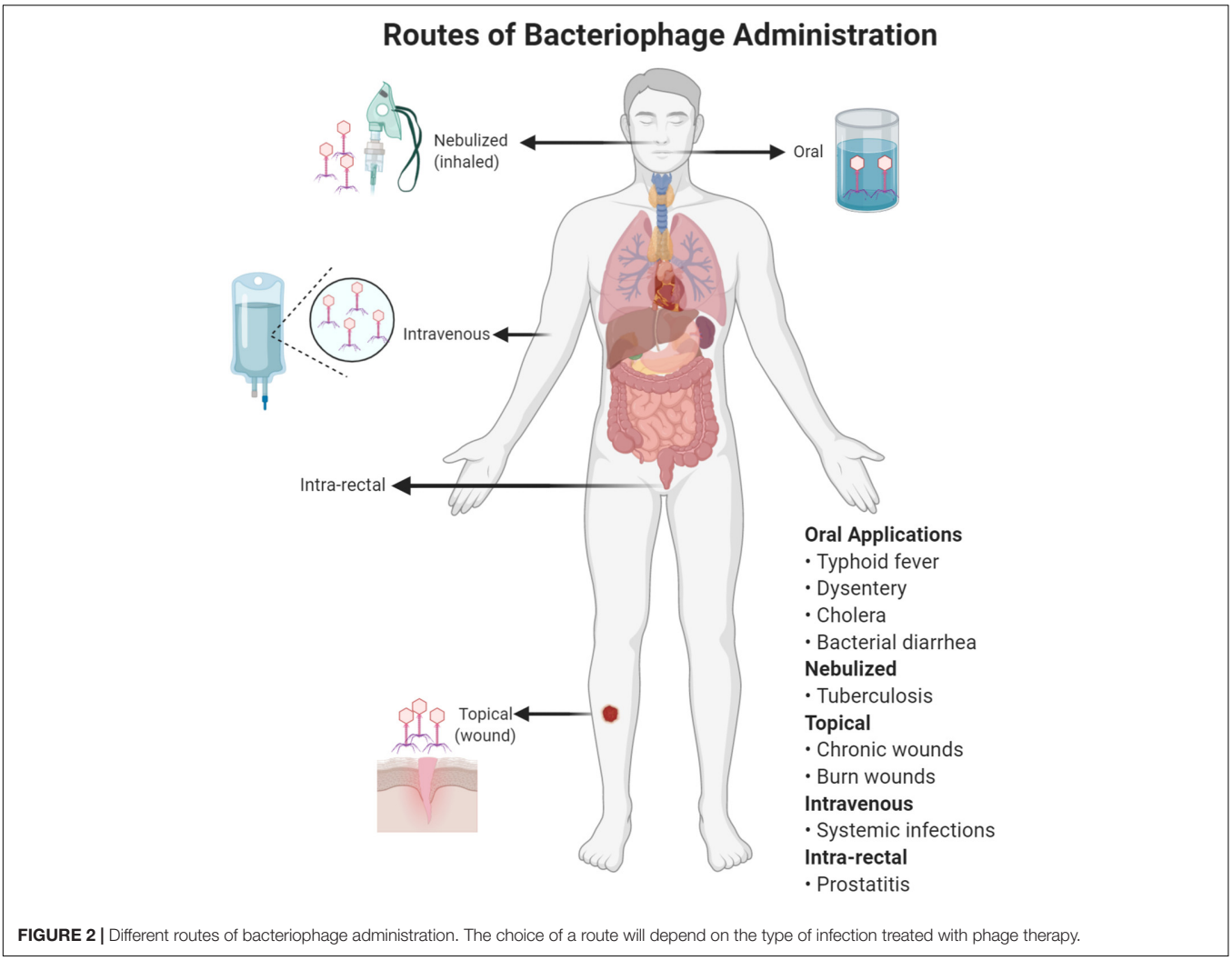

FIGURE 2 | Different routes of bacteriophage administration. The choice of a route will depend on the type of infection treated with phage therapy.

intestinal perforation (Khan et al., 2013). Case-fatality rates as high as 28% have been reported in children (Tickell et al., 2017).

Shigella is highly infectious, with very low infectious dose (DuPont et al., 1989) and efficient transmission through fecal-oral route and in contaminated food, water and fomites (**Figure 3**). Common sources include salads, poultry, milk and dairy products, seafood and vegetables (Ahmed and Shimamoto, 2015) and *Musca domestica*, the common housefly with an affinity for human excrement, has also been incriminated as a mechanical vector (Cohen et al., 1991). In humans, maternal immunity may reduce incidence of *Shigella* infections in the first 6 months of life before immunity matures and becomes protective (Mani et al., 2016). Repeated infections, however, are not unusual because immunity is highly specific and multiple serotypes cause infection. This is most problematic in developing countries during summers and rainfall season (**Figure 3**) and where over-crowding and unsatisfactory hygienic is common (Puzari et al., 2018).

The four serologically distinguishable *Shigella* species are *S. dysenteriae, S. flexneri, S. boydii,* and *S. sonnei*, all of which cause shigellosis. *Shigella* causes diarrhea among travelers and military personnel from high-income countries (Khalil et al., 2018) and it is increasingly problematic in men who have sex with men communities (Heiman et al., 2014; Baker et al., 2018). Geographical variations and changing *Shigella* epidemiology (Tickell et al., 2017) makes management more difficult (Baker et al., 2018). There are several promising candidate in different stages of development (Walker, 2015) but no licensed vaccines are presently available.

Antimicrobial treatment can shorten the disease course, prevent complications and limit spread of infection through fecal shedding (Williams and Berkley, 2018). As with other endemic infections however, the treatment of shigellosis is complicated by increasing AMR. Currently, WHO recommends fluoroquinolones as first line treatment for all cases of dysentery and third-generation cephalosporins (ceftriaxone) are reserved as a second line or alternative option (WHO, 2005a) because there is high prevalence of resistance against ampicillin, tetracyclines, and sulphonamides. Quinolone, macrolide and third generation cephalosporin resistance is now increasingly widespread (Azmi et al., 2014; Chiou et al., 2016; Chung The et al., 2016) and horizontal transfer of resistance determinants between *Shigella*

TABLE 1 | Important endemic bacterial infections in developing countries and their possible phage therapy solution.

Disease	*Incidence (in millions)	MDR/XDR	Phage trials		Phage solution	
			Animals	Humans	Prophylaxis	Treatment
Typhoid	11–20	Yes/Yes	Yes	Yes	Yes	Yes
Cholera	2.86	Yes/No	Yes	Yes	Yes	Yes
Shigellosis (dysentery)	>80	Yes/Yes	Yes	Yes	Yes	Yes
Tuberculosis	10	Yes/Yes	Yes	No	Yes	No
Acute bacterial diarrhea other than dysentery and cholera	∼5–5.5	Yes/No	Yes	Yes	Yes	Yes

**The references for these data can be found in relevant sections.*

and related Enterobacteriaceae may be contributing to the alarming increase in frequency of MDR globally (Mandomando et al., 2009; Chang et al., 2011; Gu et al., 2012; CDC, 2013; Aggarwal et al., 2016; Nuesch-Inderbinen et al., 2016; Hussen et al., 2019; Wang et al., 2019; Houpt et al., 2020).

Phages against *Shigella flexneri* may offer an alternative—they have been shown to prevent epithelial cell adhesion and invasion of phage-specific strains as well as other isolates of same species in a human intestinal organoid-derived infection model (Llanos-Chea et al., 2019). Early studies in mouse models showed phages can reach a range of anatomic sites including the brain (Dubos et al., 1943) and that phage therapy delayed up to 4 days can still prevent mortality (Morton and Engley, 1945).

Effective phages are relatively easily isolated, including from environmental water sources during dysentery outbreaks (Doore et al., 2018) and there is a rich history of phage therapy for shigellosis, with large and successful interventions reported since the 1930s (Chanishvili, 2012; Goodridge, 2013). Phages have been used successfully for dysentery prophylaxis (Babalova et al., 1968; Anpilov and Prokudin, 1984) and as biocontrol agents in water (Jun et al., 2016) and food (Zhang et al., 2013; Soffer et al., 2017).

Acute Bacterial Diarrhea Due to *E. coli*

Enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), and diffusely adherent *E. coli* (DAEC) are distinguished on the basis of specific virulence properties (Croxen et al., 2013). EPEC and ETEC are endemic in developing countries where ETEC strains are a major cause of traveler's diarrhea while STEC causes large outbreaks around the world and, like Shigella, may be complicated by haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Rojas-Lopez et al., 2018). The more than 6 billion cases and 1.5 million deaths in all age groups from diarrheal illness globally (Gregory et al., 2018; Spencer et al., 2018) include both viral and bacterial etiologies, but ETEC is in the top 10, accounting for more than 50,000 deaths in 2016, predominantly in young children in sub-Saharan Africa and South Asia (Khalil et al., 2018).

There is a steady rise in antibiotic resistance among diarrheagenic *E. coli* with many developing countries reporting >70% of isolates to be MDR (Nguyen et al., 2005; GebreSilasie et al., 2018; Zhang et al., 2018). The WHO does not recommend routine use of antimicrobials to treat diarrhea where it is not possible to initially distinguish between etiological agents, because their efficacy is limited (WHO, 2005b) and in order to minimize selection for resistance (Laxminarayan et al., 2013). An oral, live attenuated recombinant vaccine (ACE527) was shown to generate strong immune response against ETEC in human volunteers (Darsley et al., 2012; Harro et al., 2019) but is not yet widely available.

Phage preparations have been used to treat potentially lethal enteropathogenic *E. coli* infection in calves, piglets and lambs (Smith and Huggins, 1982, 1983; Smith et al., 1987) and a large Phase I/II trial for the treatment of pediatric *E. coli* diarrhea established safety of orally administered phages in children but failed to significantly improve symptoms or outcomes (Sarker et al., 2016). Inadequate strain coverage, gastric acid neutralization and low pathogen density (for phage amplification) may be contributors to these unsatisfactory outcomes (Brussow, 2019) and need to be considered in future study designs.

Foodborne Diseases

Foodborne diseases (FBD) are defined as any illness caused by the ingestion of contaminated food or drink. The FBD disease spectrum ranges from gastrointestinal symptoms (most common) to debilitating chronic conditions including neurological and immunological disorders as well as multi-organ failure, cancer, and death (Grace, 2015). Until recently, data on the incidence of FBD and its associated costs were mostly limited to high-income countries as many developing countries lack reliable data on the incidence of FBD.

The annual global incidence of FBD was recently estimated to exceed 600 million cases and 420,000 deaths annually (WHO, 2015), largely experienced in low-middle income countries (LMICs) with less well developed food safety and regulatory and reporting systems (Grace, 2015). Total productivity loss is estimated at more than US$95 billion in LMICs with another $15 billion USD spent on treatment (World Bank Group, 2018) and biological pathogens are the most important food safety risks in developing countries (Käferstein, 2003). Rapid urbanization is a key factor in developing countries with marked increases in food establishments and outlets numbers, inadequate knowledge in food handlers, lack of clean water, use of untreated human and animal waste in agriculture, suboptimal refrigeration, and poor personal hygiene (Käferstein et al., 1997).

Salmonella, Campylobacter, Enterohemorrhagic E. coli (EHEC) and *Listeria* are among the most common bacterial pathogens

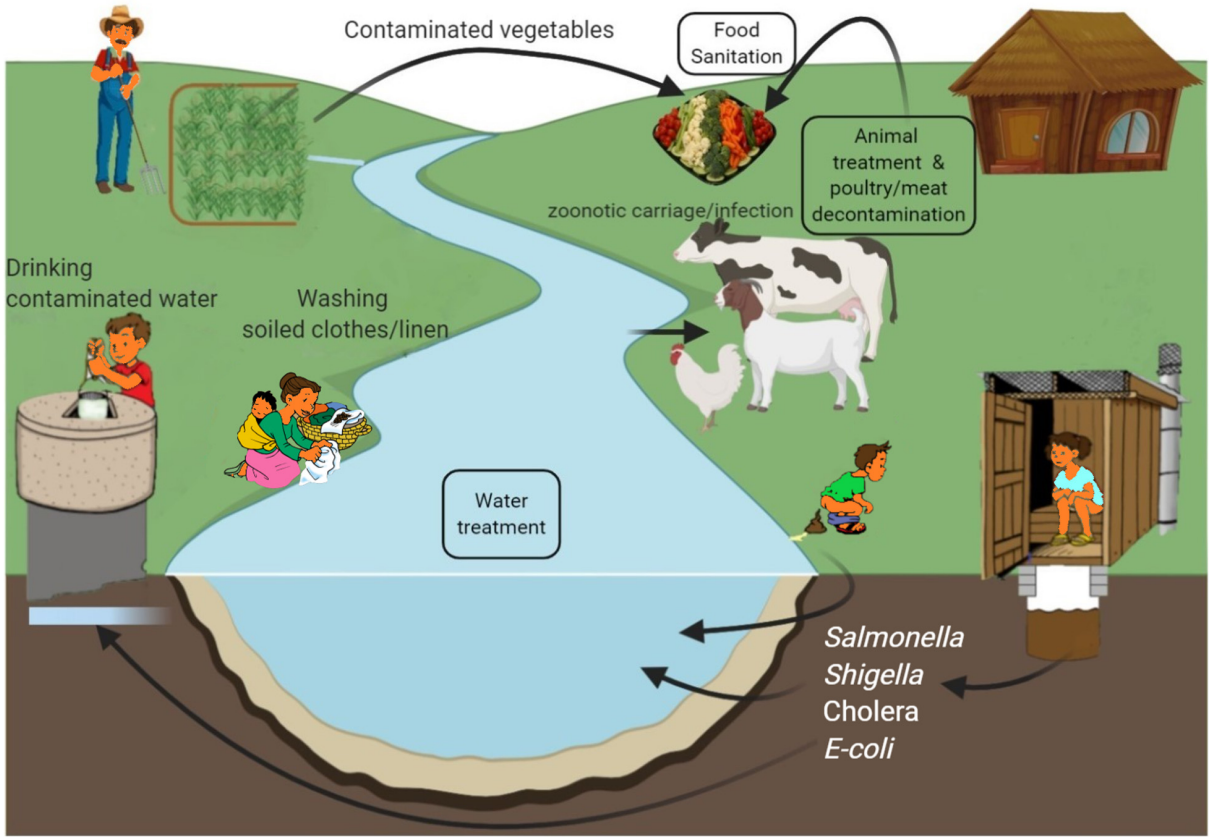

FIGURE 3 | Environmental sources of common bacterial infections in developing countries and possible phage interventions (boxed text) as preventive measure.

implicated in FBD (Havelaar et al., 2015). Multiple interventions are used to reduce contamination of foods with biological pathogens (Vikram et al., 2020) but all have limitations. There is an increased need for "natural" antimicrobial alternatives for food decontamination and preservation and many studies have established the usefulness of phages, including decontamination of processing surfaces (Moye et al., 2018). In addition to the appeal as an "organic" choice, phages have the advantages of high specificity, self-replication, co-evolution with their bacterial hosts, low toxicity, relatively inexpensive manufacturing and high tolerance of the conditions common in food processing and storage (Sillankorva et al., 2012).

Phages have long been recognized as a normal part of the food ecosystem (Whitman and Marshall, 1971; Hudson et al., 2005). *Listeria monocytogenes,* often associated with fresh or minimally processed foods such as dairy products and salads, was an early target for food sanitization attempts and their effectiveness in food products is well described (Amy Strydom, 2015). The first commercial phage biocontrol application against *Listeria* contamination for food safety was approved by FDA in 2006 (ListShield^{TM}) and a Generally Recognized as Safe (GRAS) designation was issued to another *Listeria*-specific phage biocontrol product (Listex^{TM}) later that same year. Since then, many other phage applications have been introduced into the market successfully (Vikram et al., 2020) and other pathogens such as *Campylobacter* may soon follow (Sillankorva et al., 2012). There are approved food safety phage applications against *Salmonella*, *enterohaemorrhagic E- coli* and *Shigella* (Moye et al., 2018), which constitute the majority of foodborne diseases of bacterial origin. Food sanitation by phage biocontrol should remain a high priority research and development agenda.

Tuberculosis

Tuberculosis (TB) is an airborne infectious disease caused by *Mycobacterium tuberculosis* complex. It is primarily a disease of the lungs but can disseminate to affect other parts of the body. TB is a leading cause of global mortality and the highest among all infectious diseases, including HIV/AIDS. WHO reported an estimated 10 million new cases of TB with 1.2 million deaths in HIV-negative people and more than 250,000 deaths among HIV-positive people in 2018 (WHO, 2019). Developing countries account for more than two thirds of the global incidence of TB, with most high-income countries reporting less than 10 cases per 100,000 population annually compared to 150–400 incident cases per 100,000 in high TB burden countries (WHO, 2019). Malnutrition, crowded living and work conditions and a lack of access to diagnosis and treatment contribute to a continued high disease burden in poor countries (Lonnroth et al., 2009). TB incidence is declining slowly at 1.6% per year globally but isoniazid and now rifampicin-resistant TB is

increasingly reported (GBD Tuberculosis Collaboratoras, 2018) and the WHO's "End TB Strategy" target of >4% sustained annual decline in incidence of new cases by 2,030 seems unlikely to be met (Furin et al., 2019).

Effective antimicrobial treatment for TB typically includes at least an intensive initial 2 months of therapy with four first-line drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) followed by continuation phase with isoniazid and rifampicin for 4 months (Nahid et al., 2016; WHO, 2017b), the efficacy of which is usually monitored with repeated sputum smears, cultures and chest X-rays. Drug toxicity is not uncommon and the duration of therapy makes compliance difficult (Pai et al., 2016). MDR TB is steadily increasing in endemic countries (Dheda et al., 2017) and the emergence of extremely drug resistant TB (XDR-TB), for which there is almost no effective treatment, creates a pool of patients actively transmitting untreatable strains (Pietersen et al., 2014). The Bacillus Calmette-Guerin (BCG) vaccine is used worldwide mainly to prevent life-threatening tuberculosis in infants and young adults but is not an effective eradication strategy (Abubakar et al., 2013). There are other candidate vaccines in development with better efficacy profile in newborns and children, as well as adolescents and adults (Pai et al., 2016), but none are widely available.

There is considerable interest in mycobacteriophages for treatment and control of TB. They have been tested against MDR and XDR strains (Hatfull, 2014) but early animal studies met with mixed results (Mankiewicz and Beland, 1964; Sula et al., 1981) and there are no human trials as yet. some benefits were observed in guinea pigs with disseminated TB (Zemskova and Dorozhkova, 1991) but there are concerns regarding phage penetration to attack intracellular bacilli or those deep within granulomatous lesions (Hatfull, 2014). Use of non-virulent bacteria to deliver phage payloads into macrophages led to a significant reduction in viable intracellular bacilli in experimental animals (Broxmeyer et al., 2002) but the clinical applicability of this approach needs further evaluation.

Prospects for phage prophylaxis of TB contacts may be better. Inhaled bacilli from exposure should be easily accessible to phages introduced directly into the lungs in high concentrations, perhaps reducing risk of resistance evolution among small populations of targeted bacteria (Vehring, 2016) and this approach appeared to reduce the pulmonary MTB burden in mice up to 3 weeks after challenge (Carrigy et al., 2019). Aerosolised phage delivery into the lungs may be the optimal route (reviewed by Abedon, 2015) but particle diameter and tolerance of physical, osmotic and thermal stress are important considerations (Hoe et al., 2013).

The natural course of TB is indolent and subclinical with most disease transmission in high-burden countries unrecognized until recrudescent (secondary) disease develops, often decades later. Phage therapy may have value as a prophylactic regimen in recent exposure populations but studies would probably need to be randomized as adjunctive therapy (with standard agents) and surrogate (e.g., serological) markers may be needed to avoid decades of follow-up. Phage prophylaxis and therapy may be most valuable in severe and/or XDR-TB where need is urgent and response relatively easily measured.

DISCUSSION

Phage therapy is increasingly re-emerging as a viable therapeutic option against serious bacterial infections. Notwithstanding the long experience in parts of Europe and the numerous anecdotes of successful phage therapy for human infection (d'Herelle, 1931; Summers, 2001; Abedon et al., 2011; McCallin and Brüssow, 2017; Gordillo Altamirano and Barr, 2019), it remains poorly accepted in Western medicine.

Recently, there has been a noticeable increase in compassionate use of phage therapy to treat serious bacterial infections and the results are promising (Maddocks et al., 2019; Aslam et al., 2020; Cano et al., 2020; Petrovic Fabijan et al., 2020b). However, robust clinical trials are very few and mostly unsuccessful (Sarker et al., 2016; Jault et al., 2019; Leitner et al., 2020).

There are some important challenges to the progress of phage therapy through the existing regulatory frameworks, most prominent being the scarcity of essential data from human therapy. Phage(s) selection, optimal route of administration and dosage, the relative benefit of single vs. multiple phages and/or combinations with antibiotics all remain as questions that have yet to be decided. Pharmacokinetic and pharmacodynamic properties are generally regarded as unpredictable and must be better defined (Nilsson, 2019). The *in vivo* co-evolution of phages and their target bacteria and the potential interference of human immune system further complicate phage therapy, and the fact that compassionate use cases typically utilize phage/s as adjuvants to antibiotic/s makes it more difficult to attribute efficacy. The emergence of phage-resistant bacteria may be countered by using multiple effective phages in a cocktail (Pirnay and Kutter, 2020) and this popular approach means that complex multi-phage dynamics must also to be considered.

Oral administration is an appealing option, especially for enteric diseases, with the key advantage of greater simplicity of manufacture. Safety of phage therapy has been well demonstrated for suitable preparations but oral bioavailability and dosing kinetics are not well understood (Bruttin and Brussow, 2005; Sarker et al., 2017; Petrovic Fabijan et al., 2020b). The limited data that are available suggest that bioavailability may be adequate for oral dosing in animals (Watanabe et al., 2007; Miedzybrodzki et al., 2017) and humans (Weber-Dabrowska et al., 1987).

Aerosol delivery of phages into lungs is also relatively simple (Abedon, 2015; Maddocks et al., 2019) but GMP-grade preparation for nebulization (as for IV administration) remains a financial and logistical challenge.

Much of the current demand for phages to treat major developing country diseases relies on goodwill to meet it. Organizations such as "Phages for Global Health" raise awareness and educate/train laboratory and healthcare staff and build capacity in developing countries while biotech ventures such as "PhagePro" work to develop phage-based products to treat cholera.

The willingness of phage laboratories in developed countries to donate therapeutic candidate phages are a boon for physicians in developing countries and organizations such as

Phage Directory[1] work to facilitate access to these but a sustainable approach must be developed.

Coordinated efforts are needed from international health organizations to properly evaluate the potential role of phage therapy and identify potential candidates for properly designed trials.

AUTHOR CONTRIBUTIONS

AK, RCYL, and JRI conceived and drafted the manuscript. All authors read and approved the final manuscript.

[1] https://phage.directory/

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Characterization and Genomic Analysis of PALS2, a Novel *Staphylococcus* Jumbo Bacteriophage

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Staphylococcus aureus is an important human pathogen that can be frequently encountered in clinical and food-processing surroundings. Among the various countermeasures, bacteriophages have been considered to be promising alternatives to antibiotics. In this study, the bacteriophage PALS2 was isolated from bird feces, and the genomic and biological characteristics of this phage were investigated. PALS2 was determined to belong to the *Myoviridae* family and exhibited extended host inhibition that persisted for up to 24 h with repeated bursts of 12 plaque-forming units/cell. The complete genome of PALS2 measured 268,746 base pairs (bp), indicating that PALS2 could be classified as a jumbo phage. The PALS2 genome contained 279 ORFs and 1 tRNA covering asparagine, and the majority of predicted PALS2 genes encoded hypothetical proteins. Additional genes involved in DNA replication and repair, nucleotide metabolism, and genes encoding multisubunit RNA polymerase were identified in the PALS2 genome, which is a common feature of typical jumbo phages. Comparative genomic analysis indicated that PALS2 is a phiKZ-related virus and is more similar to typical jumbo phages than to staphylococcal phages. Additionally, the effective antimicrobial activities of phage PALS2 suggest its possible use as a biocontrol agent in various clinical and food processing environments.

Keywords: *Staphylococcus aureus*, jumbo phage, antimicrobial agent, antibiotic resistance, genome

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INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium that causes skin infections, respiratory tract infections, and food poisoning in animals and the human body (Lowy, 1998; De Lencastre et al., 2007). Staphylococcal infection can be life-threatening if not treated immediately. Moreover, treatment of the infection has become more difficult because of the emergence of antibiotic-resistant bacteria. In particular, methicillin-resistant *S. aureus* (MRSA) is more challenging than other resistant bacteria, as it is resistant to the entire classes of beta-lactam antibiotics and even to multiple classes of non–beta-lactam antibiotics (Chambers and DeLeo, 2009; Turner et al., 2019). Additionally, MRSA ranked high on the World Health Organization's priority list for the

development of new antibiotics for antibiotic-resistant bacteria (Khan et al., 2019). These findings highlight the need to devise a new strategy to combat *S. aureus*, and there has been increasing interest in the development of alternative antimicrobial agents employing bacteriophages (phages) (Foster, 2004).

Phages are a type of virus that specifically infects bacteria, and they have several potential advantages over traditional antibiotics, such as specificity to target bacteria, capacity to self-replicate, and safety. A phage follows one of two life cycles, lytic and lysogenic cycles, and the phages that obligatorily follow the lytic cycle are better candidates for therapeutic use. Lytic phages that infect their hosts rapidly replicate and produce many progenies and ultimately lyse the bacteria, which are preferred in comparison to the phages that integrate their genome into the bacterial genome (Ghannad and Mohammadi, 2012; Altamirano and Barr, 2019; Cui et al., 2019; Reuter and Kruger, 2020).

Genomic sequence analysis revealed that phages are diverse in their genome size. Iyer et al. (2021) classified phages into three groups: small phages with genome sizes less than 100 kb, medium-sized phages with genome sizes less than 180 kb, and phages with genomes larger than 180 kb. The majority of phages reported to date have genome sizes less than 200 kbp referred to as small-genome phages, and phages with genome sizes above 200 kbp but below 500 kbp have mostly been classified as jumbo phages (Yuan and Gao, 2017). The large genome size of jumbo phages enables many proteins that rarely or do not exist in small-genome phages to be encoded, and these proteins are more complex than those existing in small-genome phages. For instance, several small-genome phages such as phage T7 have its own RNAP, but it is a single subunit RNAP, whereas most jumbo phages encode a multisubunit RNAP (Sokolova et al., 2020). This feature makes the jumbo phages generally less dependent on the host metabolism and eventually leads them to have a wider host range compared to the phages with smaller genomes (Mesyanzhinov et al., 2002). Accordingly, these attributes make jumbo phages ideal for phage therapeutic application (Guan and Bondy-Denomy, 2020). However, jumbo phages have been rarely isolated by classical phage isolation method because of the large size of virions. Jumbo phages have mostly been isolated from Gram-negative bacteria, and only 11 jumbo phages have been reported from Gram-positive bacteria to date, most of which are *Bacillus*-infecting phages and one *Staphylococcus* infecting phage, S6 (Uchiyama et al., 2014; Lood et al., 2020). *Staphylococcus* Jumbo phage S6 belongs to *Myoviridae* with genome size of approximately 270 kbp, but its genome has not been sequenced. Besides, the isolated jumbo phages contain a variety of genes with unknown functions, indicating that the jumbo phage genomes represent extremely high genetic diversity and that their functional biological characteristics have not been fully elucidated. Therefore, further research is required to obtain more fundamental understanding of jumbo phages.

In this study, we isolated a novel staphylococcal jumbo phage, PALS2 (accession no. MN091626), which has a genome size of 268,748 base pairs (bp). We investigated the morphology, host infectivity, and bioinformatics characteristics of this phage. Interestingly, PALS2 exhibited multiple small bursts to efficiently generate progeny phages and presented several common genomic features of the jumbo phages. Considering the strong bacterial inhibitory ability and broad host range of PALS2, this phage could be utilized in the development of a novel potential biocontrol agent (Hyman, 2019).

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in **Table 1**. *S. aureus* human isolate strain 93 was used for the isolation and propagation of the phage PALS2. All staphylococcal and

TABLE 1 | Antimicrobial spectrum of phage PALS2 against different bacterial species.

Bacterial host[a]	PALS2[b]
Staphylococcal strain	
Staphylococcus aureus RN 4220	T
S. aureus Newman	C
S. aureus ATCC 13301	C
S. aureus ATCC 23235	T
S. aureus ATCC 33586	T
S. aureus ATCC 33593	C
S. aureus ATCC 6538	C
S. aureus ATCC 29213	C
S. aureus ATCC 12600	T
S. aureus ATCC 25923	I
S. aureus ATCC 27664	T
MRSA CCARM 3793	C
MRSA CCARM 3089	T
MRSA CCARM 3090	T
Staphylococcus haemolyticus ATCC 29970	C
Staphylococcus epidermidis ATCC 35983	I
MRSE CCARM 3787	I
MRSE CCARM 3789	I
Staphylococcus hominis ATCC 37844	C
Staphylococcus warneri ATCC 10209	T
Staphylococcus xylosus ATCC 29971	I
Staphylococcus saprophyticus ATCC 15305	T
Staphylococcus capitis ATCC 35661	C
Staphylococcus cohnii ATCC 29974	C
Other Gram-positive bacteria	
Enterococcus faecalis ATCC 29212	–
Bacillus cereus ATCC 14579	–
Bacillus subtilis ATCC 23857	–
Listeria monocytogenes ATCC 19114	–
Gram-negative bacteria	
Salmonella enterica serovar Typhimurium SL 1344	–
Escherichia coli MG1655 ATCC 47076	–
Cronobacter sakazakii ATCC 29544	–
Pseudomonas aeruginosa ATCC 27853	–

[a]*ATCC, American Type Culture Collection.*
[b]*C, clear plaque; T, turbid plaque; I, turbid lysis zone without single plaques (**Supplementary Figure 1**); –, no lytic effect.*

enterococcal strains were grown in tryptic soy broth (TSB), and other bacteria were grown in Luria–Bertani (LB) broth at 37°C.

Bacteriophage Isolation and Propagation

Bacteriophage PALS2 was isolated from bird feces. Bird fecal samples were collected in Seoul, South Korea. A sample mixed with 50 mL of sodium chloride (NaCl)–magnesium sulfate (SM) buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 8 mM $MgSO_4{\cdot}7H_2O$) was poured into a stomacher filter bag and homogenized. From the other side of the filter in the bag, the sample was transferred to a 50 mL tube and centrifuged at 10,000 × *g* for 5 min at 4°C. After centrifugation, the supernatant was filtered using a 0.22 μm polyethersulfone (PES) membrane filter to remove bacterial cells. Then, 5 mL of the filtrate was added to an equal volume of 2 × TSB broth and subcultured with the host strain at 37°C with shaking at 220 revolutions/min (rpm) for 12 h. After incubation, the culture was centrifuged, and the supernatant was filtered as described above. The presence of phage in the filtrate was confirmed by spotting 10 μL of 10-fold serially diluted filtrates onto soft agar (TSB containing 0.4% agar) containing 100 μL of host *S. aureus* culture. The plates were incubated overnight at 37°C, and the formation of plaques was monitored. Based on the typical plaque morphology of the jumbo phage, small plaques were picked with a sterile tip and eluted in 100 μL SM buffer (Serwer et al., 2007; Lewis et al., 2020). The plaque isolation and elution steps were repeated more than three times to purify a single phage; phages were incubated at 30°C.

For propagation of the phage, TSB was first inoculated with the host *S. aureus* strain and incubated at 37°C with shaking at 220 rpm until it reached an OD_{600} of 0.25. Subsequently, $CaCl_2$ and $MgCl_2$ (at final concentrations of 5 mM each) and phages at a multiplicity of infection (MOI) of one were added followed by a 4 h incubation at 30°C. The propagated phages were centrifuged at 15,000 × *g* for 5 min, and the supernatant was filtered using a 0.22 μm PES filter to remove bacterial debris. To prepare the phage at a high titer, filtered phages of 40 mL were mixed with 5 g of polyethylene glycol 6000 and 5 mL of 5 M NaCl using a two-dimensional shaker at 4°C overnight. Phages were then precipitated by centrifugation (15,000 × *g*; 20 min; 4°C), and the pellet suspended in SM buffer was subjected to a four-layer CsCl gradient (1.3, 1.45, 1.5, and 1.7 g/mL). After ultracentrifugation at 25,000 × *g* for 2 h at 4°C, the separated phage was dialyzed against 1 L of dialysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM NaCl, and 10 mM $MgCl_2$) using a dialysis membrane tube for 2 h with a buffer change.

Morphological Analysis by TEM

Staphylococcus phage PALS2 was analyzed using transmission electron microscopy (TEM). A phage suspension [1×10^{10} plaque-forming units (PFUs)/mL] was placed on a carbon-coated copper grid and negatively stained with 2% uranyl acetate (pH 4.0). The sample was examined with an energy-filtering TEM at an operating voltage of 120 kV (Kwiatek et al., 2012). PALS2 was identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses (Lefkowitz et al., 2018).

Bacterial Challenge Assay

Tryptic soy broth (50 mL) was inoculated with the host strain *S. aureus* human isolate 93 and incubated at 37°C until the early exponential growth phase was reached. The culture was subsequently infected with the phage at MOIs of 1 and 0.1. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) each hour after phage infection, with no measurements being taken between the 7th and 16th hours (Park et al., 2012). An uninfected culture was used as a negative control.

Host Range Analysis

The bacterial strains listed in **Table 1** were incubated overnight at 37°C. One hundred microliters of each bacterial culture was mixed with 5 mL of soft agar (TSB containing 0.4% agar) and overlaid on tryptic soy agar plates. Subsequently, 10 μL of serially diluted phage PALS2 lysates (tenfold, 10^{10} to 10^3 PFUs/mL) was spotted onto the prepared plates and incubated at 30°C overnight. After incubation, the infectivity was determined based on the appearance of the spots: "C," clear single plaques; "T," turbid single plaques; "I," inhibited growth without single plaques; "—," no lysis nor growth inhibition.

Receptor Analysis

To verify the receptor, *S. aureus* RN4220, a strain free of prophages (Kreiswirth et al., 1983), type I restriction mechanisms, and capsules (Wann et al., 1999), was utilized (**Table 1**). It is known that most genetic manipulation of *S. aureus* is confined to strain RN4220 because RN4220 is defective of type I restriction–modification (RM) system, whereas different lineages of *S. aureus* contain a unique pair of type I RM systems (Cooper et al., 2017). We obtained an RN4220Δ*tagO*:erm mutant, which lacks the peptidoglycan-anchored wall teichoic acid (WTA) (Swoboda et al., 2010), and its complemented strain carrying the p*StagO* plasmid. This plasmid was constructed by subcloning the *tagO* gene into an *Escherichia coli–S. aureus* shuttle expression vector, pND50 (Brückner, 1992). Afterward, 10 μL of 10-fold diluted phage PALS2 lysate (10^{10} to 10^3 PFUs/mL) was spotted from top–left to bottom–right on soft agar (TSB containing 0.4% agar), beginning with the highest titer, with the wild-type RN4220, the RN4220Δ*tagO*:erm mutant, and the *tagO*-complemented strain.

One-Step Growth Curve

A one-step growth curve analysis was performed as described previously (Lu et al., 2003). Briefly, phage was mixed with the *S. aureus* host strain in the early exponential growth phase (5.6×10^7 colony-forming units/mL) at an MOI of 0.1. After incubation at 37°C for 5 min to enable adsorption of the phage, it was centrifuged at 10,000 × *g* for 5 min. The pellet containing infected cells was resuspended in 50 mL of fresh TSB and incubated at 37°C with shaking at 220 rpm. Samples were collected every 10 min for 6 h. Subsequently, each sample was centrifuged at 16,000 × *g* for 1 min, and the sample titer was assessed by spotting tenfold serially diluted supernatants on a double-layer agar plate. Latent period and burst size were analyzed according to the resulting titer. The latent period of phage was calculated by measuring the interval of time between

phage infection and the initial burst of phage titers. The burst size of phage was calculated by dividing the phage titers at postburst plateau phase by the initial phage titers.

Phage DNA Extraction

Bacteriophage genomic DNA was purified as previously described (Wilcox et al., 1996). Prior to purification, the phage lysate was treated with DNase and RNase for 30 min at room temperature to remove bacterial nucleic acid contaminants. To degrade the phage capsid, phage lysates were then treated with lysis buffer containing 20 mM EDTA, 50 μg/mL proteinase K, and 0.5% sodium dodecyl sulfate for 1 h at 56°C. Phenol–chloroform–isoamyl alcohol (25:24:1) was added to the mixture at a 1:1 ratio and was gently inverted. The resulting sample was centrifuged at 5,000 rpm for 5 min. The top aqueous layer was collected and treated with a 1:1 mixture of equilibrated phenol and chloroform at a 1:1 ratio. The solution was mixed, centrifuged, and isolated as described above. These steps were repeated after treating the isolated top solution with chloroform. Finally, ethanol precipitation was performed. The DNA pellet was washed with 100% ethanol and resuspended in TE buffer.

Full Genome Sequencing and Bioinformatics Analysis

Purified PALS2 phage genomic DNA was sequenced using the Illumina MiSeq system and assembled with the *de novo* assembly algorithm of CLC Genomics Workbench 10.0.1 at Sanigen Inc., South Korea. Open reading frames (ORFs) were predicted using the Glimmer v3.02 (Delcher et al., 2007) and GeneMarkS (Besemer et al., 2001) software packages. The ORFs were annotated using the Rapid Annotation using Subsystem Technology pipeline, as described previously (McNair et al., 2018). This annotation was further complemented using BlastP (Altschul et al., 1997) and Cognizer (Bose et al., 2015). The complete genome sequence of *Staphylococcus* phage PALS2 was deposited in GenBank under the accession number MN091626.

To map phage PALS2 onto the phage population network, vContact2 (Jang et al., 2019) was utilized, which extracts predicted proteins from each viral genome to build viral protein clusters (PCs), which are subsequently utilized to calculate genome similarities between pairs of viruses. This approach was utilized because viruses lack a universal gene marker. In fact, the coding genes for terminase large subunits, which could have played potential roles as phylogenetic markers because of their relative conservedness to other phage proteins, were not predicted to be present in the genome of phage PALS2. Briefly, the phage PALS2 genome was processed using VirSorter (v.1.03) and vContact2-Gene2Genome (v.1.1.0) on the CyVerse cyberinfrastructure (Merchant et al., 2016) prior to using vContact2. Next, using NCBI Bacterial and Archeal Viral RefSeq (v.88) as the reference database, protein sequences were subjected to all-to-all BlastP searches with an *E* value threshold of 10^{-4} and were defined as homologous PCs in the same manner as previously described (Bolduc et al., 2017). Based on the number of shared PCs between the genomes, vContact2 calculated the degree of similarity as the negative logarithmic score by multiplying the hypergeometric similarity *p* value by the total number of pairwise comparisons. Subsequently, pairs of closely related genomes with a similarity score of ≥1 were grouped into viral clusters (VCs) using ClusterONE (Nepusz et al., 2012) equipped in the vContact2 tool. The resulting network was visualized with Cytoscape (v.3.7.2) using an edge-weighted spring embedded model, which places the genomes or fragments sharing more PCs closer to one another.

RESULTS AND DISCUSSION

Morphological Characteristics of Phage PALS2

The new *S. aureus*–infecting phage PALS2 was isolated from bird feces. This phage formed relatively small clear plaques (<1 mm on 0.4% softer agar) on the lawn of *S. aureus* human isolate 93 as a bacterial host strain, indicating that PALS2 possesses a characteristic common to large phages, such as jumbo phages (Saad et al., 2019). A previous study observed that virion diffusion of large phages is retarded on a top agar layer, resulting in the formation of small plaques (Gallet et al., 2011).

Transmission electron microscopy analysis of purified PALS2 demonstrated that the phage possesses an icosahedral head with a non-flexible and contractile tail, indicating that this phage belongs to the *Myoviridae* family. The diameters of the head measured approximately 94 nm in width and 101 nm in height. The contractile tail was 201 ± 29 nm in length (n = 4 phages) (**Figure 1**). The basal tuft extending from the baseplate also supported that this phage has the morphotype of a myovirid (O'Flaherty et al., 2005). Of more than a hundred jumbo phages reported to date, approximately 90% have been classified as *Myoviridae* (Yuan and Gao, 2017; Imam et al., 2019).

Bacterial Challenge Assay of Phage PALS2

A bacterial challenge assay was performed in liquid culture to determine the bacterial growth inhibition by phage PALS2. The complete inhibition of host cells by PALS2 was observed 1.5 or 2 h after infection with MOI 1 or 0.1, respectively, and persisted for up to 24 h after infection with both MOIs (**Figure 2**). These results demonstrate that PALS2 has a strong bacterial inhibitory ability against its target host bacteria. *S. aureus* phages SA97 and SA exhibited bacterial growth inhibition of up to 10 and 8 h, respectively, at MOI 1 (Chang et al., 2015; Hamza et al., 2016). Other *S. aureus* phages UPMK_1 and UPMK_2 inhibited bacterial growth for 2–3 h at MOI 1 (Dakheel et al., 2019). PALS2 also showed a relatively fast bacterial inhibitory effect compared to the other jumbo phages that have been characterized. For example, bacterial inhibition was only observed until 200 min after infection by *Pseudomonas aeruginosa* phage MIJ3 (Imam et al., 2019) and 11 h after infection by *Cronobacter sakazakii* phage CR5 (Lee et al., 2016). Therefore, these results suggest the possibility of phage PALS2 as an effective *Staphylococcus* countermeasure (**Figure 2**).

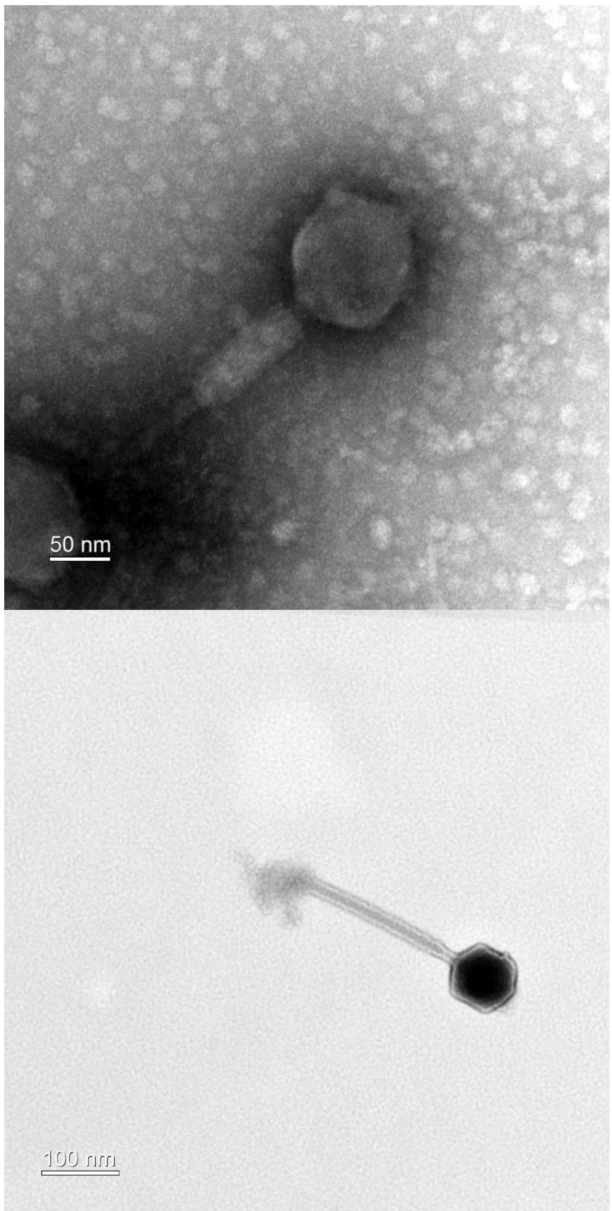

FIGURE 1 | Transmission electron microscopy of phage PALS2. The phage belongs to the family *Myoviridae*.

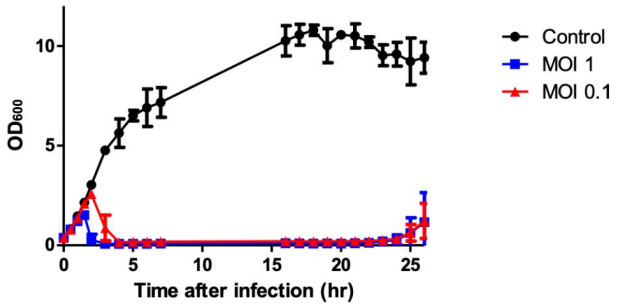

FIGURE 2 | Bacterial growth inhibition assay against *S. aureus*. Cells were prepared in three groups: the control group without phage (●) and the experimental group with phage (■, ▲; MOIs of 1 and 0.1, respectively). The data shown are the mean values from three independent measurements, and the error bars represent the standard deviations.

Host Range of Phage PALS2

To determine the antimicrobial spectrum of phage PALS2, 14 strains of *S. aureus*, 11 strains of staphylococcal strains other than *S. aureus*, and four additional Gram-positive strains were analyzed, as shown in **Table 1**. Notably, PALS2 was able to infect all *S. aureus* strains tested, including MRSA strains. Phage PALS2 was also capable of infecting other staphylococcal strains, including *S. haemolyticus*, *S. hominis*, *S. warneri*, *S. saprophyticus*, *S. capitis*, and *S. cohnii*. The wide antimicrobial spectrum of phage PALS2 suggests its potential applicability as a biocontrol agent in clinical settings, but further research is required to determine a more comprehensive host range of PALS2 against *S. aureus* strains by identifying phage resistance mechanisms in a hierarchical manner.

Receptor Analysis of Phage PALS2

It is known that peptidoglycan-anchored WTAs of *S. aureus* serve as receptors for *S. aureus*–targeting phages. A previous study suggested that most *Staphylococcus* myoviruses require WTA to infect host bacteria (Xia et al., 2011). Therefore, we tested the infectivity of PALS2 on the Δ*tagO* mutant of *S. aureus* (RN4220Δ*tagO*:erm) (Kaito and Sekimizu, 2007), which lacks WTA (**Figure 3**). RN4220Δ*tagO*:erm was determined to be resistant to PALS2 (**Figure 3B**), and phage susceptibility was recovered when the strain was complemented with the *tagO* gene (**Figure 3C**; Park et al., 2010). These results indicated that WTA is the host receptor of phage PALS2.

One-Step Growth Curve of Phage PALS2

The latent period and burst size of PALS2 were determined by one-step growth curve analysis with the host strain *S. aureus* human isolate 93 (Hyman and Abedon, 2009). The latent period of PALS2 was 30 min followed by a small burst of 12 PFUs/infected cell (**Figure 4**). Multiple bursts of the same size occurred after an additional latent period of 30 min and were repeated until the phage concentration reached 10^9–10^{10} PFUs/mL. Consistent with this result, multiple burst phenomena have also been observed in other phages (Shkoporov et al., 2018; Sharma et al., 2019). *Erwinia* jumbo phage Deimos–Minion showed double bursts of approximately five PFUs/infected cell, and *Bacteroidales* phage ΦcrAss001 showed two small bursts of 2.5 PFUs/infected cell. These phages commonly have small burst sizes, which could provide an explanation for the multiple bursts exhibited by jumbo phages, including PALS2. A previously reported study demonstrated that the small amounts of phages that come out of initially infected cells proceed to infect other intact cells in the culture, resulting in a second lag period and a subsequent burst (Ellis and Delbruck, 1939).

The small single burst size of PALS2 indicates low phage productivity, but the phage replication capacity is expected to be

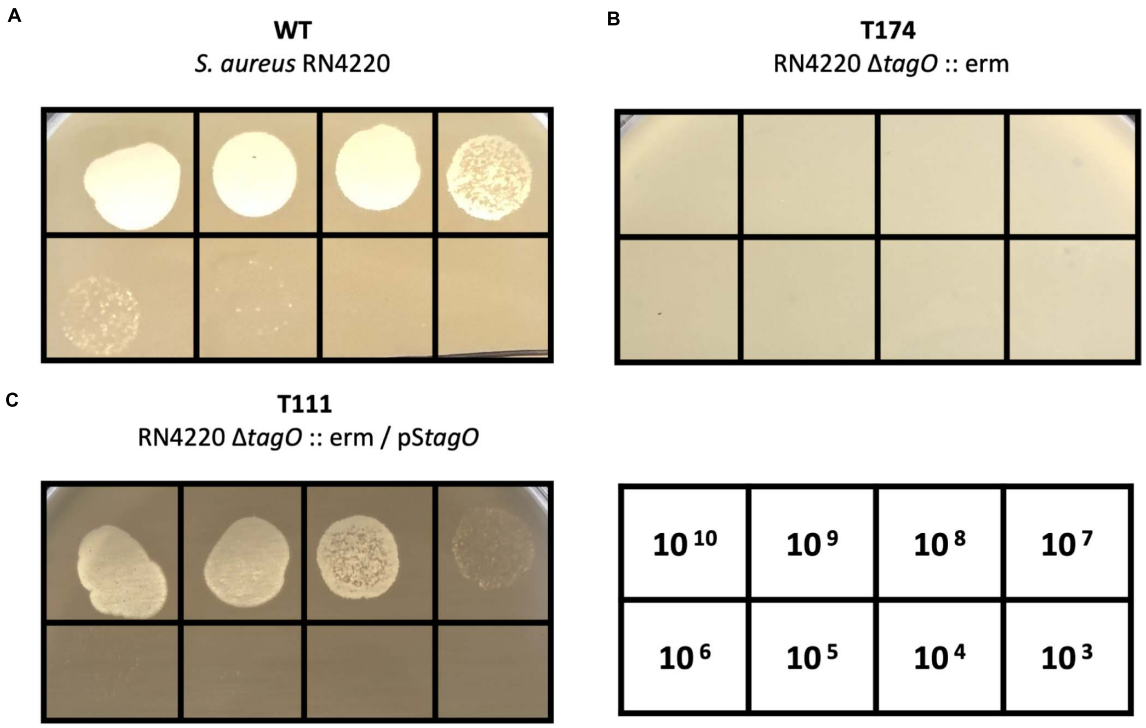

FIGURE 3 | WTA-dependent infection of *S. aureus* phage PALS2. Phage PALS2 lysate was spotted onto lawns of **(A)** wild-type RN4220, **(B)** Δ*tagO*:erm mutant, T174, and **(C)** *tagO*-complemented strain, T111. The numbers in the table indicate the titer (PFUs/mL) of phage PALS2 spotted on the plate. Plaque formation indicates successful adsorption and infection by phage PALS2.

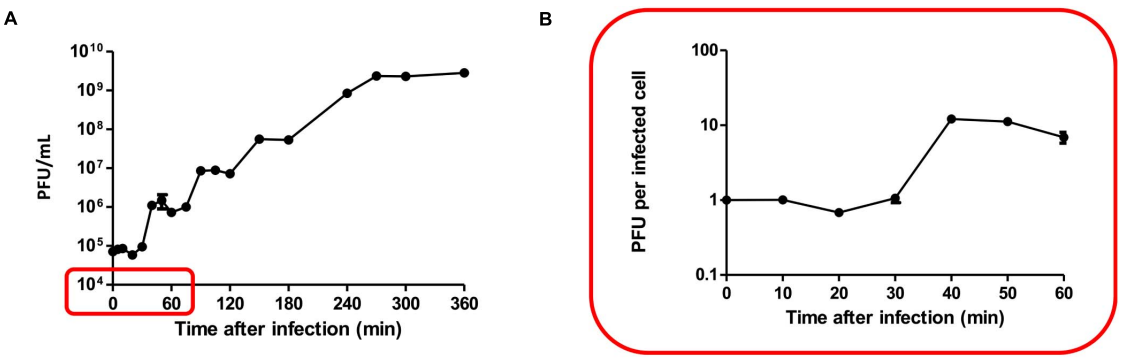

FIGURE 4 | One-step growth curve. The data shown are the mean values from four independent measurements, and the error bars represent the standard deviations. **(A)** Multiple bursts of roughly the same size occurred approximately 30 min after each burst until the phage concentration reached its maximum, 10^9–10^{10} PFUs/mL. **(B)** PALS2 demonstrated a latent period of 30 min followed by a small burst of progeny (12 PFUs per infected cell).

redeemed through repeated bursts. The high replication capacity of PALS2 could also be verified in the bacterial growth inhibition assay, where inhibition of *S. aureus* persisted for up to 24 h after infection with MOI 0.1, which is the same MOI condition given in the one-step growth curve assay.

Whole Genome Analysis of Phage PALS2

Genomic features of phage PALS2 were elucidated by whole-genome analysis. PALS2 is a double-stranded DNA virus with a 268,748-bp-long chromosome and is thus classified as a jumbo phage (Yuan and Gao, 2017). The average G + C content of the genome is 24.66%, and it contains 279 ORFs that could encode proteins and one gene encoding tRNA with asparagine anticodon. The majority of the predicted genes (232 ORFs) encoded hypothetical proteins with unknown functions (**Supplementary Table 1**). No genes of predicted lysogeny functions, such as integrase, transposase, excisionase, repressor, and genome attachment site (attP), were identified in the genome of PALS2, suggesting that PALS2 is a lytic phage (Altamirano and Barr, 2019). Additionally, genes related to bacterial toxicity

were not identified, but PALS2 encoded a metal-dependent hydrolase containing a metallo–beta-lactamase superfamily motif (PALS2_034). BlastN analysis demonstrated that the whole-nucleotide sequence of PALS2 shares less than 1% average nucleotide identity with other publicized phage genomes. Based on the low-genome homology between jumbo phages and other phages, many of them have been classified as a new lineage (Yuan and Gao, 2017). It is assumed that PALS2 is also a new phage species that does not belong to any other phage group.

The large genome size of jumbo phages is believed to have evolved through the acquisition of additional functional genes (Yuan and Gao, 2017). PALS2 had 14 genes involved in DNA replication and repair and five genes responsible for nucleotide metabolism (**Figure 5**). Interestingly, four paralogous genes of the RNA polymerase (RNAP) subunit (PALS2_067, PALS2_089, PALS2_188, and PALS2_228) were identified in the PALS2 genome and were annotated as DNA-directed RNAP β, β′, ω, and δ subunits, respectively. When searched against the NCBI database, the annotated RNAP β, β′, and δ subunits showed significant biological similarities to those of various jumbo phages. The RNAP subunits of *Bacillus* phage AR9 exhibited the highest similarities with those of PALS2 (Lavysh et al., 2016): PALS2_067 had 91% coverage with 38% identity; PALS2_089 had 93% coverage with 37% identity; and PALS2_228 had 97% coverage with 32% identity. Meanwhile, the annotated RNAP ω subunit showed no detectable similarity to any protein in the NCBI database. Overall, it is assumed that the gene expression of PALS2 may be dependent more on its own RNAPs than on the host bacterial RNAPs (Ceyssens et al., 2014; Leskinen et al., 2016). This finding is also a probable explanation for the broad host range of PALS2.

PALS2 also encoded an additional gene involved in the lysis of host cells. This feature is expected to facilitate the infection ability of jumbo phages (Gill et al., 2012). Two genes (PALS2_239 and PALS2_240) were predicted as endolysins, which contribute to the hydrolysis of bacterial peptidoglycan. Endolysin is generally composed of two functional domains: enzymatically active domains (EADs) at the N-terminal position and cell wall–binding domains (CBDs) at the C-terminal position (Loessner, 2005). According to Pfam32.0 analysis, PALS2_239 was predicted to contain a cysteine, histidine-dependent amidohydrolases/peptidase domain (pfam05257; *E* value, 1.38e-07) for EAD and an SH3_5 domain (pfam08460; *E* value, 4.06e-08) for CBD. PALS2_240 was predicted to be composed of an amidase 2 domain (pfam01510; *E* value, 5.87e-20) for EAD and an SH3_5 domain (pfam08460; *E* value, 1.25e-08) for CBD.

Finally, PALS2 has an atypical genome arrangement, which is a common feature of jumbo phages. The genomes of small-genome phages such as a well-studied phage T4 are usually clustered into functional modules, and phage genes are expressed in a timely manner for the production of progeny phages. However, jumbo phages lack recognizable modular genome characteristics that help to classify this group of phages (Imam et al., 2019). Genes of associated functions in the genome of PALS2 were scattered or only formed subclusters, indicating that the majority of the genes are not expressed in a time-dependent manner but rather under control of the phage-encoded RNAPs (Leskinen et al., 2016).

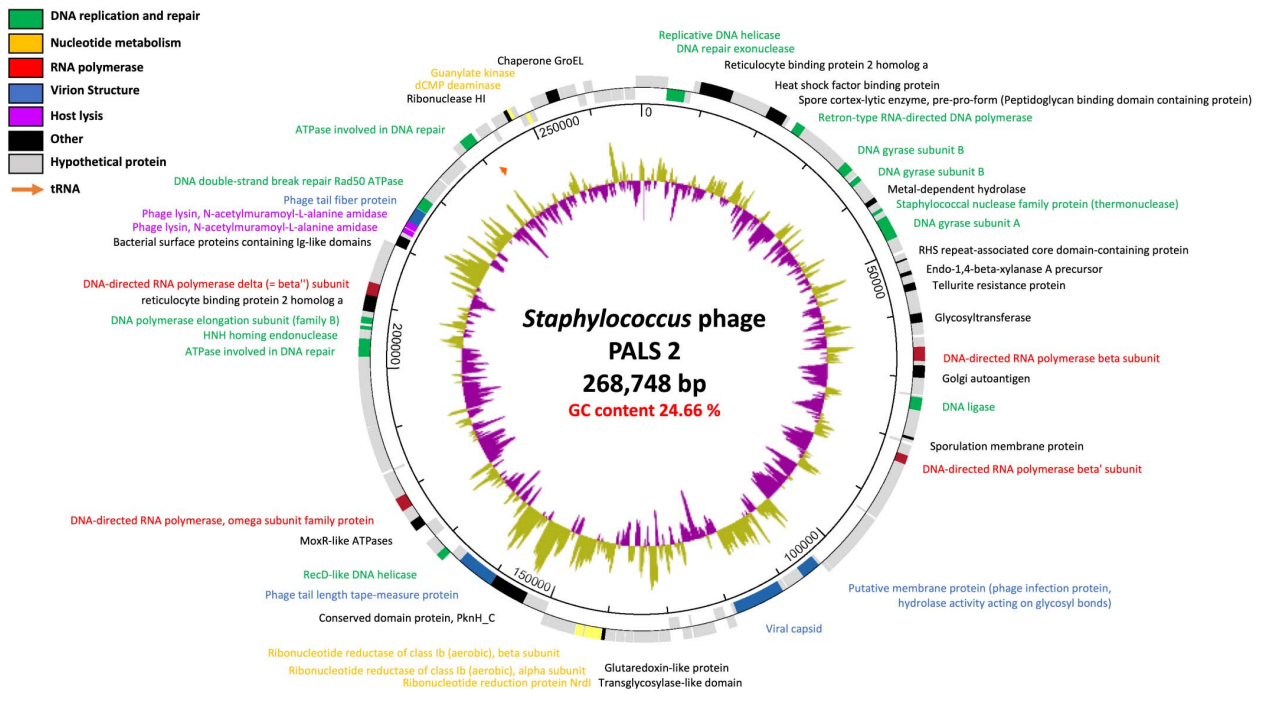

FIGURE 5 | Genome map of phage PALS2. Predicted ORFs are arranged on the PALS2 genome. Functional groups are categorized into colors as shown in the legend. The inner track represents the GC content plot (500-base window) of the phage.

Overall, the genomic characteristics of PALS2 suggest that the large genome of this phage has evolved toward reduced reliance on the host bacterium. Accordingly, this genomic evidence, indicating a broad host spectrum and efficient lysis ability, suggests that PALS2 can be used as an effective therapeutic agent in the future.

Comparative Genomics of Phage PALS2

To determine the location of PALS2 within the phage continuum context, we conducted a gene-sharing network analysis using vContact2 (Jang et al., 2019). This approach localized viruses sharing a high number of genes (homologous PC) into a VC. In the visualized network, the nodes indicate viral genomes, and the edges between nodes indicate the gene content similarities between each paired genome. Subnetworks that did not contain jumbo phages were removed from the gene sharing network to simplify the visualization, resulting in 1,446 nodes and 52,066 edges (**Figure 6A**). A full list of viral genomes used in the vContact2 analysis can be found in **Supplementary Table 2**. Of 53 jumbo phages identified on the map, 28 were placed in the largest subnetwork, including PALS2, and 25 others were placed into two isolated subnetworks. Conventionally, jumbo phages are classified primarily into T4-related or phiKZ-related phage groups. In the resulting map, the majority of jumbo phages in the largest subnetwork were related to the T4-like phages (**Figure 6A**). Notably, PALS2, which is placed in the large subnetwork, was distant from the T4-like phage group. The map indicated that PALS2 is connected to four *Myoviridae* phages: two *Staphylococcus* phages, Twort and phiIPLA-C1C, *Bacillus* jumbo phage AR9, and *Yersinia* phage jumbo phiR1-37 (**Figure 6B**). According to the similarity score estimated with the hypergeometric equation (Bolduc et al., 2017), PALS2 was more strongly connected to the jumbo phages than to the *Staphylococcus* viruses. This finding indicates that PALS2 shares more genes with the jumbo phages than the two staphylococcal phages. The result of the comparative genomic analysis using vContact2 explains the acquisition of many new genes as PALS2 evolved into a jumbo virus and thus suggests that PALS2 is more similar to typical jumbo phages.

In a previous study, phages AR9 and phiR1-37 were reported to be phiKZ-related, given that they possess genes encoding proteins homologous to the subunits of cellular RNAPs (Lavysh et al., 2016). The RNAPs of phiKZ-related phages are distinct from other phage RNAPs in that they are evolutionarily related to cellular RNAPs and belong to the double-psi beta-barrel fold family of polymerases (Yakunina et al., 2015). RNAPs of phiKZ-related phages, however, are not equal to their cellular counterparts, as they do not harbor the RNAP α subunit, nor

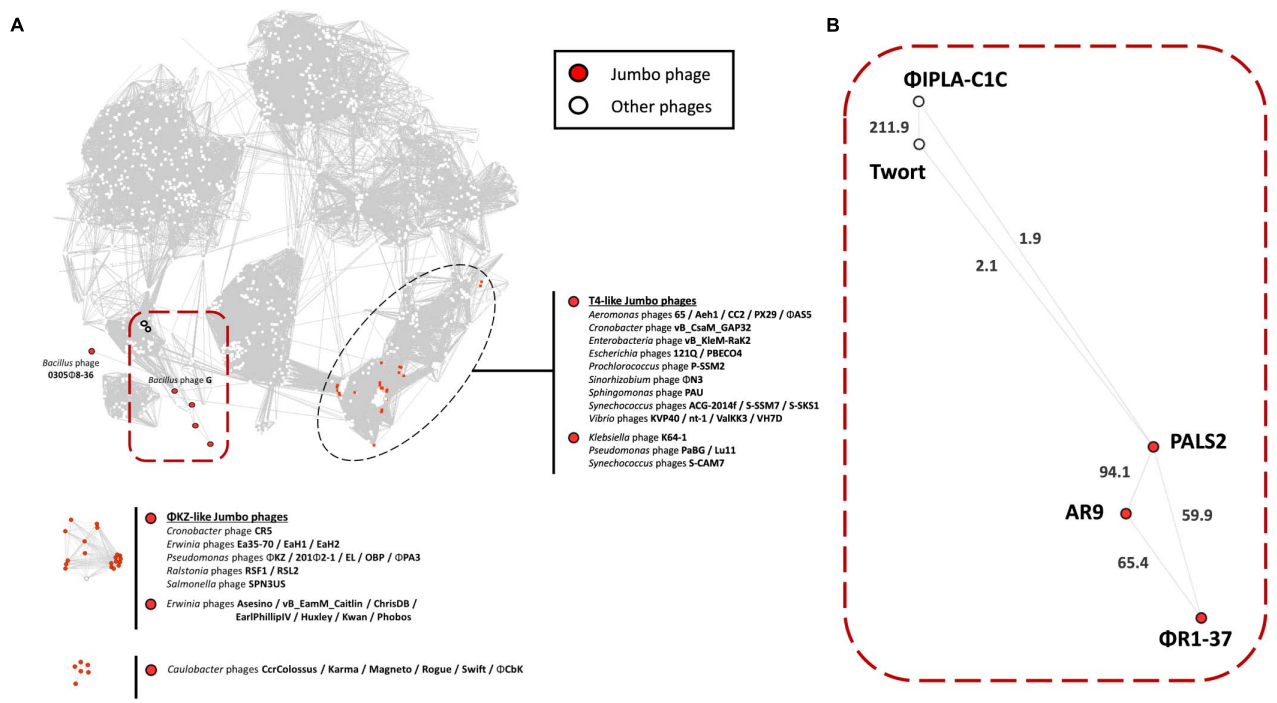

FIGURE 6 | Protein-sharing network of phage PALS2. **(A)** A network representation was produced using Cytoscape. Nodes indicate phage genomes, and edges between two nodes indicate their statistically weighted pairwise similarities with phage–phage similarity scores of ≥1. Nodes are colored according to the phages' characteristics: jumbo phage, red; other phages, white. Jumbo phages were further labeled with their designated names. On the top is the largest subnetwork that contains the largest number of phage genomes, including jumbo phages from the T4-like clade. The other subnetworks below are the smaller clusters of jumbo phages and their relatives that were disconnected from the largest subnetwork. Phage PALS2 and its first neighbors are situated in the large subnetwork, which is highlighted with a red square boundary. **(B)** Enlarged view of phage PALS2 and its relatives. Edge length is proportional to the similarity values estimated with the hypergeometric equation. The values are written on top of each edge. The data indicate that PALS2 is closer to jumbo phages AR9 and phiR1-37 than to the staphylococcal phages Twort and phiPLA-C1C.

do the phages encode bacterial sigma factors. BlastP analysis demonstrated that PALS2 encodes a unique multisubunit RNAP, and each subunit, except for PALS2_188, is significantly related to phages AR9 and phiR1-37, suggesting that it is possible that PALS2 is also a phiKZ-like virus.

CONCLUSION

This research elucidated the physiological and genomic characteristics of a novel *Staphylococcus* jumbo phage PALS2. PALS2 exhibited a wide host range spectrum covering many species of *Staphylococcus* including MRSA. Therewith, the strong bacterial inhibitory activity of PALS2 demonstrated that the phage itself can potentially serve as an effective biocontrol agent. Genome exploration demonstrated that PALS2 possesses many extra functional genes, such as RNAP subunits and an extra lysis protein, which may have strengthened the infection ability of PALS2 and broadened its host spectrum. These observations place PALS2 among candidate phages to study their therapeutic potential in fighting infections with staphylococci. Further research regarding phage–host interactions would allow better insight into this jumbo phage.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YL, BS, YC, and SR conceived and designed the experiments. YL carried out the main body of research, performed the experiments and bioinformatics analysis, and wrote the manuscript. BS contributed in performing the phage characterization experiment and wrote the manuscript. YC contributed in isolating and characterizing the phage. SR supervised the work progress and edited the manuscript. 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: https://www.frontiersin.org/articles/10.3389/fmicb.2021.622755/full#supplementary-material

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Analysis of Selection Methods to Develop Novel Phage Therapy Cocktails Against Antimicrobial Resistant Clinical Isolates of Bacteria

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Antimicrobial resistance (AMR) is a major problem globally. The main bacterial organisms associated with urinary tract infection (UTI) associated sepsis are *E. coli* and *Klebsiella* along with *Enterobacter* species. These all have AMR strains known as ESBL (Extended Spectrum Beta-Lactamase), which are featured on the WHO priority pathogens list as "critical" for research. Bacteriophages (phages), as viruses that can infect and kill bacteria, could provide an effective tool to tackle these AMR strains. There is currently no "gold standard" for developing a phage cocktail. Here we describe a novel approach to develop an effective phage cocktail against a set of ESBL-producing *E. coli* and *Klebsiella* largely isolated from patients in United Kingdom hospitals. By comparing different measures of phage efficacy, we show which are the most robust, and suggest an efficient screening cascade that could be used to develop phage cocktails to target other AMR bacterial species. A target panel of 38 ESBL-producing clinical strains isolated from urine samples was collated and used to test phage efficacy. After an initial screening of 68 phages, six were identified and tested against these 38 strains to determine their clinical coverage and killing efficiency. To achieve this, we assessed four different methods to assess phage virulence across these bacterial isolates. These were the Direct Spot Test (DST), the Efficiency of Plating (EOP) assay, the planktonic killing assay (PKA) and the biofilm assay. The final ESBL cocktail of six phages could effectively kill 23/38 strains (61%), for *Klebsiella* 13/19 (68%) and for *E. coli* 10/19 (53%) based on the PKA data. The ESBL *E. coli* collection had six isolates from the prevalent UTI-associated ST131 sequence type, five of which were targeted effectively by the final cocktail. Of the four methods used to assess phage virulence, the data suggests that PKAs are as effective as the much more time-consuming EOPs and data for the two assays correlates well. This suggests that planktonic killing is a good proxy to determine which phages should be used in a cocktail. This assay when combined with the virulence index also allows "phage synergy" to inform cocktail design.

Keywords: antimicrobial resistance, antibiotic resistance, urinary tract infection, bacteriophage, phage therapy, ESBL, *E. coli*, *Klebsiella*

INTRODUCTION

Antimicrobial resistance (AMR) is a major global challenge. It is part of the key target priorities for several prominent organisations including the World Health Organisation (WHO), European Centre for Disease Prevention and Control (ECDC) and National Institute of Health Research (NIHR) (Tacconelli et al., 2018). It has been predicted that more people will die of AMR infections than cancer by 2050 and AMR associated deaths are estimated to be approximately 10 million people per year (O'Neill, 2014). AMR has been compounded by a reduction in novel antibiotic discovery, the persistent use of antibiotics and thus, the rapid emergence of bacterial strains that are resistant to both existing and new antibiotics (Tacconelli et al., 2018). The most clinically relevant group of multi-drug resistant (MDR) pathogens are referred to collectively as the ESPAKEE organisms (Gram-positive *Enterococcus faecium* and *Staphylococcus aureus*, as well as Gram-negative *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae*, *Enterobacter* species, and *Escherichia coli*), and are together responsible for the majority of hospital-acquired infections (Pendleton et al., 2013). Urinary tract infections (UTIs) are prevalent and can cause serious infections *per se* but can also act as infection sources for sepsis (urosepsis) and septicaemia. The majority of organisms associated with urosepsis are *E. coli*, which are responsible for 50% of cases, and *Klebsiella* along with other *Enterobacter* species, which total 15% of cases (Kalra and Raizada, 2009). Furthermore biofilm formation has been shown to be crucial in infections such as catheter-associated UTIs with both *E. coli* and *Klebsiella* (Hancock et al., 2010).

Extended Spectrum Beta Lactamases (ESBL) are plasmid-mediated enzymes that, if expressed by a bacterial strain, confer resistance to antibiotics containing a beta-lactam ring in their molecular structure such as penicillins, cephalosporins, and carbapenems (Sykes and Matthew, 1976; Livermore, 1987; van Duin and Doi, 2017). ESBL-producing strains of both *E. coli* and *Klebsiella* have been detected from a variety of sources worldwide (Bush, 2018). They pose a serious global public health threat due to the difficulties associated with treatment of infections with ESBL-producing bacteria. Although, Sakellariou et al. (2016) reported no difference in mortality rates of infections caused by either ESBL-producing *E. coli* (23.8%) or ESBL-producing *Klebsiella* (27.1%), they did report that septicaemia associated with ESBL-producing *Klebsiella* has a higher morbidity (sepsis with organ failure).

The decline in antibiotic discovery and emergence of resistance to last line antibiotics (Pendleton et al., 2013), motivates the need for alternative antimicrobials. A promising solution is the therapeutic application of lytic bacteriophages (phages), which are viruses that kill bacteria. Phage therapy has a long history of use in countries such as Georgia, Poland, and France (Kutateladze and Adamia, 2008; Ansaldi et al., 2018; Gorski et al., 2018) where it has been used alongside or instead of antibiotics to treat bacterial infections for more than 100 years. There is a critical need to widen access to this therapy, either as an alternative or supplement to antibiotic treatment. If phage therapy is to be developed in the Western world, it is advisable to focus on bacterial diseases for which no other treatments exist and those which have high levels of AMR (Tomas et al., 2018).

A phage cocktail is a mixture of several phages and has two potential clinical advantages (Chan and Abedon, 2012). One is to combine the individual phages to broaden the number of strains the phages are able to infect. The second is to combat phage resistance, which can occur with the use of single phages. By using a cocktail of phages, strains that become resistant to one phage can potentially be targeted by other phages within the cocktail. In the context of the current study, the primary goal for the phage cocktail was to provide a broader host range than any of the individual phages alone. Host range coverage was prioritised over efficiency of killing with regards to the phage cocktail selection. This is because in a clinical context, it would be beneficial to provide partial treatment to a wider number of patients, allowing synergy with the immune system and antibiotics, rather than treating only a select few patients (Chan et al., 2013; Mattila et al., 2015). The overall aim was to identify phages that individually have broad host ranges and collectively when combined would cover ∼90% of the either the ESBL-producing *E. coli* or *Klebsiella* collection.

Although phage cocktails have been designed and their efficacy reported in the literature previously, there are no current guidelines to standardise the development of an optimised cocktail for antibiotic resistant bacteria or indeed to predict the efficacy of phages at least under *in vitro* conditions. Through the development of the phage cocktail in the current study, we have generated a data set that allows comparison of four different methods of assessing phage virulence (the ability of the phage to kill bacteria) across a panel of 38 bacterial isolates. These tests are: Direct Spot Test (DST), Efficiency of Plating (EOP) assay, a planktonic killing assay (PKA) and a biofilm assay. Both the DST and EOP assay are frequently utilised in the determination of phage virulence (Mirzaei and Nilsson, 2015) and both tests use the double agar plate method. The DST is a reasonably good method for initial host range screening, but it does not provide a reliable indication that the phage can replicate on the host strain. The EOP assay indicates productive infection of the host strain by the phage from which the efficiency of infection of the host can be determined. The PKA was assessed as an alternative to the labour-intensive DST and EOP approaches. This method monitors the optical density of a liquid culture of bacteria to which a phage combination was added using a plate reader over 24 h. The previous three methods examine the virulence of phages based on killing bacteria under "normal" growth conditions *in vitro* and so the final method chosen was a biofilm assay. This assay provides an insight into phage virulence in an *in vitro* model of infection and biofilm formation. Genomic analysis was performed on all 38 ESBL-producing clinical isolates to determine the relationships between susceptibility to phage infection and genomic content. The genetic relationship between the most sensitive and most resistant clinical isolates was determined. The final six phages selected for the ESBL phage cocktail were also sequenced to confirm suitability for phage therapy by ensuring they did not encode for any known undesirable traits (toxins/lysogeny).

This article focuses on the development of a phage cocktail that is effective against ESBL-producing *E. coli* and *Klebsiella* that

were largely isolated from patients in United Kingdom hospitals. In producing this data, we describe an efficient screening cascade to develop cocktails, which will be relevant for other target AMR bacterial species. This data shows a novel, direct comparison of results across the four phage virulence tests for individual clinical isolates and indicates that the PKAs are a reliable and time efficient way to assess phage efficacy.

MATERIALS AND METHODS

Bacterial Strains

Thirty eighty strains of ESBL-producing bacteria were examined during this study; 19 *E. coli* and 19 *Klebsiella*. All strains were clinical isolates from UTIs; 14 of the *E. coli* isolates were from Leicester Royal Infirmary, United Kingdom; 5 from Huashan Hospital, Shanghai and 19 *Klebsiella* isolates from Leicester Royal Infirmary, United Kingdom (**Supplementary Table S1**). All bacteria were grown at 37°C in either Luria-Bertani Broth (LB—Thermo Fisher Scientific, United Kingdom) at 100 rpm or on LB 1% (w/v) agar plates. All strains were stored in 50% glycerol stocks at −80°C until required. The bacterial strains were sequenced by MicrobesNG with the Standard Whole Genome Service, Illumina Sequencing by sending the bacterial strains as samples.

Phage Collection, Isolation, Amplification, and Visualisation

Phages were collated from several sources with the majority coming from collaborations with other research projects (**Supplementary Table S2**). Phages were isolated using the method previously described by Kropinski et al. (2009). To identify phages, 100 μl enrichment, 100 μl culture and 3 ml LB 0.5% (w/v) agar were poured onto a LB 1% (w/v) agar plate and incubated overnight at 37°C. Single plaques were picked and transferred to 500 μl SM Buffer [100 mM NaCl (Sigma-Aldrich, United Kingdom), 8 mm $MgSO_4$ 7 H_2O (Sigma-Aldrich, United Kingdom), 0.1% (w/v) gelatin (Sigma-Aldrich, United Kingdom), 50 mM Tris-HCl pH 7.5 (Sigma-Aldrich, United Kingdom)]. This process was repeated to give five rounds of single plaque purification and stored in SM buffer.

Phage stocks were made using the double layer agar method. Briefly, an overnight culture of the host strain was diluted 1:100 in LB and grown for 2 h to an $\sim OD_{550}$ of 0.2 at 37°C, 100 rpm. 500 μl of the bacterial culture and 200 μl of phage stock were added to 8 ml of 0.5% (w/v) LB agar and poured onto 120 × 120 mm square LB 1% (w/v) agar plates. The plates were incubated overnight at 37°C. The plates were agitated for 2 h in 10 ml SM buffer. The top layer was removed and centrifuged at 4,000 × *g* for 15 min. The supernatant was filter-sterilised through 0.2 μm pore size filters and the resultant phage stock titre was determined using double agar overlay plaque assays (Kropinski et al., 2009). Stock was stored at 4°C. Phage UP17 (vB_EcoM_UP17) was propagated using *E. coli* EA2; phage JK08 (vB_SsoM_JK08)—*E. coli* MH10; phage 113 (vB_SsoM_113)—*Shigella sonnei* B31; phage 2811 (vB_KpnS_2811)—*Klebsiella pneumoniae* KR2811; phage 311F (vB_KpnM_311F)—*K. pneumoniae* KR311; phage 05F (vB_KpnM_05F)—*K. pneumoniae* MH05.

Transmission Electron Microscopy imaging for the phages UP17, 113, 2811, 311F and 05F was performed at University of Leicester, United Kingdom. The phages were negatively stained with 1% (w/v) uranyl acetate on 3 mm carbon-coated copper grids and visualised with a JEM-1400 transmission electron microscope (JEOL UK Ltd., United Kingdom) with an accelerating voltage of 120 kV. Digital images were collected with an Xarosa digital camera (EMSIS, Germany) with Radius software for phage 113; all other phages were imaged using a Megaview III digital camera (EMSIS, Germany) instead. Imaging for phage JK08 was performed at the Max Rubner-Institut, Germany with a Tecnai 10 transmission electron microscope (FEI, Eindhoven, The Netherlands) operated at an acceleration voltage of 80 kV.

Direct Spot Testing (DST)

Bacterial cultures were grown overnight, then diluted 1/100 in LB and grown for 2 h to $\sim OD_{550}$ of 0.2. 500 μL of the culture was added to 8 ml 0.5% (w/v) LB agar kept molten at 55°C and poured onto LB 1% (w/v) agar square 120 × 120 mm plates. 20 μl of phage stock (10^9/10^{10} pfu/ml) was spotted onto the plate, left to dry and then incubated overnight at 37°C. The appearance of the spot was graded: ++++ complete lysis; +++ lysis with resistant colonies; ++ hazy lysis; + visible plaques; 0 no visible plaques (**Supplementary Tables S3, S4**).

Efficiency of Plating (EOP)

This method has been previously described by Kutter (2009); 5 mM calcium chloride was supplemented to the 0.5% (w/v) LB agar for the *E. coli* and *Shigella* phages. Plaques on each plate were counted and the relative EOP was given as the ratio between the phage titre in pfu/ml (plaque forming units/ml) for the test host strain and the titre of the propagating host strain. Propagating host for phages UP17, JK08 and 113 was *E. coli* MH10. The propagating host for 2811—*K. pneumoniae* KR2811; for 311F—*K. pneumoniae* KR311; and for 05F—*K. pneumoniae* MH05.

Planktonic Killing Assay (PKA)

Experiments were carried out using the BMG Labtech SPECTROstar Omega, using a flat bottom 96 well plate (Sarstedt, Germany). 100 μl of a 1:100 dilution of overnight cultures was added to the 96 well plate, grown to A_{600} OD 0.15 (1×10^8 CFU/ml), then 100 μl of phage cocktail (containing 1×10^8 PFU/ml of each individual phage) was added. Working with a MOI of 1:1; throughout all the experiments. Final concentrations were achieved using LB as a diluent. The microtiter plates were securely sealed using gas-permeable parafilm M (Amcor, United States). OD readings (A_{600}) were taken every 5 min for a total of 24 h with shaking 10 s prior to each reading. The microtiter plate had a positive control for every individual clinical strain for comparison (bacteria only), as well as a negative control (LB only) and 3 blanks (LB and gentamicin 10 μg/ml). Each cocktail was repeated in triplicate for each ESBL-producing clinical isolate and the data was merged to give a single killing assay curve.

The killing assay curves were analysed by a modified objective method (Storms et al., 2019) which was devised using the generated curve to give a "virulence index." The virulence index score was calculated comparing the area under the curve of the individual phage or cocktail against the positive control whilst in log phase. This virulence index was normalised to a figure between 0 and 1, 0 = not effective and 1 = highly effective.

Biofilm Assay

Bacterial cultures were grown overnight at 37°C, 100 rpm. 100 μl of 1:100 dilution in LB of each bacterial strain was added to 96 well flat bottom microtiter plate in triplicate for both controls and phage cocktail treated. The whole experiment was also repeated in triplicate for all bacterial strains. After 24 h at 37°C, the LB was removed, and each well was washed with PBS. For the controls, 100 μl of fresh LB instead of 100 μl of the final ESBL cocktail was added (10^8 PFU/ml of each individual phage). After an additional 24 h of incubation, 20μl of resazurin (0.15 mg/ml—Sigma-Aldrich) was added and incubated at 37°C. OD readings were taken at A_{595} with Labtech.com LT-4500 at 4 and 24 h post incubation.

Phage DNA Extraction

Phage lysate at titres of 10^{11} PFU/ml was used to extract DNA using a modified phenol-chloroform-isoamyl method as previously described (Nale et al., 2015). The final DNA pellet was dissolved in 5 mM Tris HCl. This method only applies to phages UP17, 113, 2811, 311F, and 05F. For phage JK08, DNA isolation was performed using the Norgen Phage DNA isolation Kit (Norgen Biotek, ON, Canada) according to the manufacturer's instructions.

Sequencing and Bioinformatic Analysis

Genome sequencing was conducted by MicrobesNG[1], which was supported by the BBSRC (grant no. BB/L024209/1) for phages UP17, 311F and 05F as well as all the bacterial genomes. *De novo* assembly of the trimmed reads using Trimmomatic 0.30 (Bolger et al., 2014) from MicrobesNG was carried out using SPAdes genome assembler 3.12.0 (Bankevich et al., 2012) with default settings.

For the bacterial genomes, contigs were annotated using Prokka v1.12 (Seemann, 2014) and the assembly metrics were calculated using QUAST 5.0.2 (Gurevich et al., 2013). MLST 2.16.2 was used for characterisation of the bacterial strains (Seemann, 2020[2]). ABRicate with Resfinder database was used with default settings to screen the genome of each strain for the presence of antimicrobial resistance and virulence genes (Zankari et al., 2012; Feldgarden et al., 2019).

Sequence data for the bacterial genomes was also used to create phylogenetic trees (**Figure 4**) using MEGA7 v7180411 (Kumar et al., 2018) and visualised using iTOL v5.5 (Letunic and Bork, 2007) based on the core genome SNPs. For phages 113 and 2811, the genomes were sequenced using an Illumina MiSeq, with a v3 kit (600 cycles). Genomic libraries were prepared using the Illumina Truseq Nano DNA library Preparation Kit as per the manufacturer's instructions. The genomes were assembled using MEGAHIT (Li et al., 2015); phage 2811 (version 1.2.1) and phage 113 (version 1.1.4). Phage termini were identified using PhageTerm v1.0.11 (Garneau et al., 2017). Phage JK08 was sequenced using an Illumina MiSeq using a v2 kit (2 × 250). Illumina Truseq PCR-free library preparation kit was used as per manufacturer's instructions for genomic library preparation. Genome assembly was performed with MIRA v4.0.2 (Chevreux et al., 1999).

The genomes of phage UP17, 311F, and 05F were assembled by subsampling reads to an approximate coverage of 100× with seqtk[3] and assembled with SPAdes v3.12.0 with only assembler option (Bankevich et al., 2012). Phage genomes were annotated as previously described (Michniewski et al., 2019). To check for antibiotic resistance and virulence genes within the phage genomes, ABRicate was used with the card and vfdb databases, respectively.

Accession Numbers

All bacterial and phage genomes were submitted to the European Nucleotide Archive (ENA) under project accession number PRJEB34549. Individual accession numbers are provided in **Supplementary Tables S1, S2**.

Statistical Analysis

GraphPad Prism 7.04 (La Jolla, CA, United States) was used for statistical analysis for the biofilm assays. The results were expressed as mean ± SEM after analysis with 2-way ANOVA. A $p < 0.05$ was considered significant.

RESULTS

Comparison of Phage Virulence Methods—DST, EOP, and PKA

The three methods used to assess phage virulence: DST, EOP, and PKA were compared. These three tests form the basis for the initial screening of a phage library to identify phages with the broadest host range. The data generated also allowed direct comparison of DST versus EOP, as these two assays are commonly used to characterise phages (Sybesma et al., 2016; Montso et al., 2019; Rivera et al., 2019; **Supplementary Tables S5, S6**).

The final three phages selected for the final ESBL cocktail based on their effectiveness against the ESBL-producing *E. coli* strains were UP17, JK08, and 113. With phages 2811, 311F, and 05F selected to target ESBL-producing *Klebsiella*. Phages were selected based on the results of the DST, EOP and PKA data (**Figure 1**). The selection of the final three phages was based on combining the minimum number of phages to have the maximal effect. For example, with the *E. coli* phages using four phages resulted in the same percentage coverage of using only three (**Supplementary Table S5**).

[1]http://www.microbesng.uk

[2]https://github.com/tseemann/mlst

[3]https://github.com/lh3/seqtk

FIGURE 1 | Summary of the ESBL-producing *E.coli* clinical isolate (n = 19) coverage of final *E.coli* phages **(A)** UP17, **(B)** JK08, and **(C)** 113 and ESBL-producing Klebsiella clinical isolate (n = 19) coverage of final Klebsiella phages **(D)** 2811, **(E)** 311F, and **(F)** 05F across the three selection tests [Direct Spot Test (DST), Efficiency of Plating (EOP) and Killing (Planktonic Killing Assay)]. Isolate coverage was determined by the following parameters: DST $\geq$ + appearance score; EOP $>$ 0.01; Killing $\geq$0.2 virulence index score.

The final three *E. coli* phages were selected on the basis of having the broadest clinical isolate coverage. The following coverage was observed: phages UP17, JK08, and 113 could lyse 14/19 (74%), 13/19 (68%), and 14/19 (74%) of *E. coli* clinical isolates, respectively (**Figure 1**). When the phages were combined based on DST data, they provided coverage of 18/19 clinical isolates (95%) (**Supplementary Table S5**). The final three phages selected to be effective against the ESBL-producing *Klebsiella* clinical isolates gave overall coverage of 17/19 (89%) based on DST data (**Supplementary Table S6**). In comparison, the individual phages gave the following coverage: phage 2811 lysed 7/19 (37%), phage 311F lysed 6/19 (32%), and phage 05F lysed 11/19 (58%) (**Figure 1**).

The DST data highlighted which clinical isolates were lysed by the phages. To determine if the phages could efficiently replicate on the clinical isolates they infected, EOP studies were conducted. A detectable EOP was defined as the ratio compared to the control stain was $>$ 0.01. Across all the phages, the number of isolates on which they could replicate within (EOP) were lower than those lysed (DST) as would be expected (**Figure 1**). Collectively for the three *E. coli* infecting phages, EOP data showed 13/19 strains (68%) compared to 18/19 (95%) predicted by the DST. DST overestimates the efficiency of killing compared to EOP and PKA. For example, UP17 only effectively replicates (EOP score of $\geq$ +) in 6/14 of the clinical isolates identified by DST.

There is a closer relationship between the PKA and EOP data; but the trend appears to be that PKA is slightly lower than EOP isolate coverage. For example, the PKA showed that phage 05F was effective (virulence index $\geq$ 0.2) for 5/19 (26%) clinical isolates compared with EOP 4/19 (21%) (**Figure 1**). Based on EOP data for phage 2811, it suggests that the phage could only replicate on 2/19 (11%) clinical isolates compared with 1/19 (5%) on the PKA (**Figure 1**).

Characterisation of the Final Six Phages Selected for the ESBL Phage Cocktail

The final phages selected to target ESBL-producing *E. coli* were UP17, JK08, 113 and for *Klebsiella* the final phages were 2811, 311F, and 05F, totalling 6 phages in the final cocktail. There was no lytic activity of the *Klebsiella* phages against the *E. coli* clinical isolates or vice-versa based on DST (**Supplementary Tables S3, S4**). The phage genomes were analysed to ensure that they did not carry genes known to allow a lysogenic lifestyle and did not contain any genes encoding for known toxins. A summary of the characteristics of the final six phages are shown in **Figure 2**.

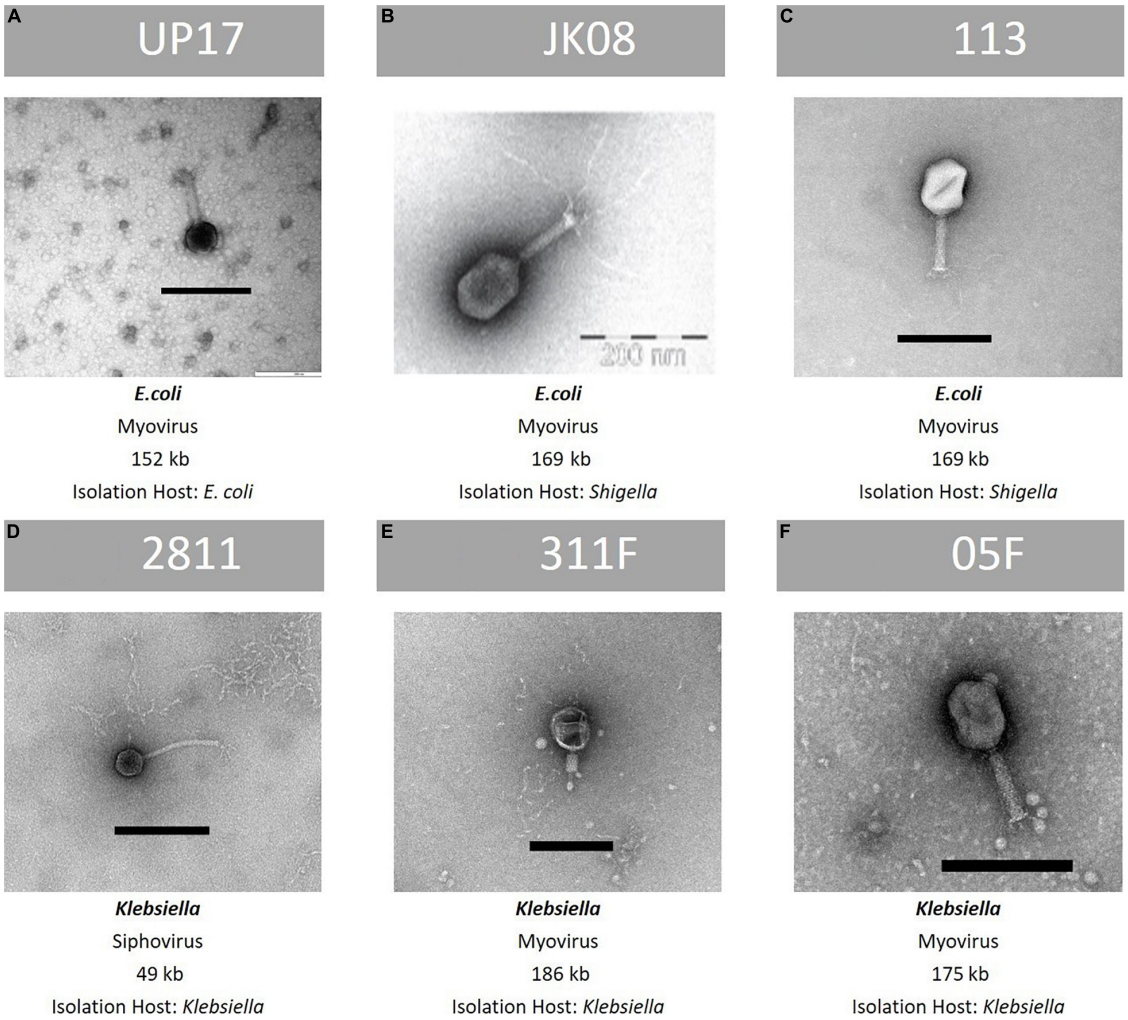

FIGURE 2 | Summary of key features of the final six phages within the ESBL cocktail—TEM image, family classification, genome size and species of propagation host. From top row left to right, **(A)**: phage UP17, **(B)**: phage JK08, **(C)**: phage 113. From bottom left to right: **(D)**: phage 2811, **(E)**: 311F, and **(F)**: phage 05F. Black bar represents 200 nm.

Use of Virulence Index Score Demonstrates Synergy Within Phage Combinations

Analysis of the Combinations of ESBL *E. coli* Phages Using Virulence Index Scores

Phages UP17, JK08, and 113 used in various combinations of doublets, triplets and also in the final ESBL six-phage cocktail were tested using the PKA. Using the quantitative virulence index scores, all data was compared (**Table 1**). Data was compared on two scales; the macroscale to analyse only the number of clinical isolates within each virulence index category and the microscale to analyse individual clinical isolate virulence index scores for each phage combination.

Based on the virulence index data from the three individual phages (UP17, JK08, and 113), 13/19 (68%) of *E. coli* isolates should be targeted. However, only 12/19 (63%) were actually targeted (**Table 1**). There was an unexpected reduction in the number of isolates killed by the triplet phage combination (63%) when compared with the final six phage combination (53%) (**Table 1**).

When comparing virulence index scores, there were no substantial differences between the triplet cocktail (UP17, JK08, and 113) and the final six phage cocktail for the majority of the individual clinical isolates (**Table 1**). However, the virulence index identified inhibitory combinations. For example, when KR2729 was treated with phage 113 alone a high virulence index score of 0.64 is obtained (**Table 1**). But when used in combination with phage JK08 (JK08 and 113), its virulence index score dropped to almost zero (0.05) (**Table 1**). When all three phages were used in combination, the high virulence index score is restored to 0.92, which could be due to phage UP17 alone (**Table 1**). This effect is only noted where phage 113 is the only phage to have a high virulence index score, but with no noticeable effect from phage JK08 (**Table 1**). The effect was not noted in combinations where both phages JK08 and 113 had medium

TABLE 1 | The virulence index scores of individual phage and various phage combinations across all the 19 ESBL-producing *E. coli* clinical isolates.

	UP17	JK08	113	UP17 JK08	UP17 113	JK08 113	UP17 JK08 113	Final
KR2728	0.93	0.03	0.04	0.91	0.90	0.10	0.90	0.91
KR2729	0.93	0.00	0.64	0.94	0.92	0.05	0.92	0.92
KR2730	0.06	0.00	0.00	0.05	0.00	0.00	0.00	0.00
KR2731	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
KR2733	0.30	0.45	0.00	0.95	0.00	0.65	0.70	0.68
MH01	0.00	0.8	0.00	0.90	0.00	0.68	0.69	0.65
MH02	0.00	0.62	0.00	0.68	0.00	0.48	0.49	0.41
MH03	0.00	0.00	0.53	0.04	0.60	0.28	0.20	0.12
MH04	0.00	0.04	0.54	0.22	0.55	0.30	0.14	0.02
MH07	0.00	0.21	0.00	0.42	0.00	0.08	0.20	0.04
MH08	0.02	0.56	0.04	0.55	0.02	0.43	0.48	0.48
MH09	0.00	0.54	0.03	0.64	0.00	0.48	0.63	0.55
MH10	0.10	0.67	0.38	0.81	0.51	0.61	0.58	0.70
MH11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04
MH12	0.05	0.03	0.00	0.02	0.00	0.00	0.03	0.00
MH13	0.05	0.03	0.08	0.09	0.05	0.05	0.09	0.08
MH14	0.18	0.83	0.47	0.87	0.62	0.72	0.79	0.80
MH15	0.11	0.77	0.06	0.8	0.00	0.82	0.79	0.80
MH16	0.06	0.12	0.06	0.17	0.07	0.07	0.02	0.03
High > 0.5	2	7	3	10	6	5	8	8
Medium 0.2–0.5	1	2	2	2	0	5	4	2
Low > 0.001 – < 0.2	8	5	6	5	3	5	4	6
None 0	8	5	8	2	10	4	3	3
Effective	3/19	9/19	5/19	12/19	6/19	10/19	12/19	10/19
(High and Medium)	16%	47%	26%	63%	32%	53%	63%	53%

Numbers highlighted in Dark Blue represent high virulence index scores and those highlighted in Light Blue represent medium virulence index scores. The rows represent each of the ESBL-producing E. coli clinical isolates used within this study. The top column represents the phage starting with individual phage on the left to progressing across the various combinations. The phages are highlighted with different colours: UP17 (Orange), JK08 (Pink), 113 (Yellow), Final (Purple). Final = all six final phage (UP17, JK08, 113, 2811, 311F, and 05F). All values represent the mean generated from triplicate experimental data. A full diagrammatic representation of this data can be seen in ***Supplementary Figure S7****. The bottom section of the table summarises the individual phages and phage combinations into categories. The rows represent the categories, high = virulence index score > 0.5, medium = virulence index score 0.2–0.5, low = virulence index score ≥ 0.001–< 0.2, none = 0. Effective is a combination of the high and medium categories, this defines effective killing by the phage combination and its clinical isolate coverage.*

or high virulence index scores. This was exemplified by clinical isolates MH10 and MH14 (**Table 1**).

Conversely synergistic interactions were also observed. Treating KR2733 with phage UP17 or JK08 results in virulence index scores of 0.3 and 0.45, respectively. However, when used in combination the virulence index increases to 0.95 (**Table 1**). A similar pattern can be seen for clinical isolates, MH01, MH10, and MH07 (**Table 1**) with this phage combination.

There is an example within the virulence index data of diminished returns with *E. coli* clinical isolate MH03. When the clinical isolate is infected with 113 alone (0.53), JK08 and 113 (0.28), all 3 *E. coli* phages (0.20), and the final 6-phage cocktail (0.12). This demonstrates that increasing the number of phages within a cocktail is not necessarily beneficial.

Analysis of the Combinations of ESBL *Klebsiella* Phages Using Virulence Index Scores

The same selection process was carried out for comparison of *Klebsiella* phages. The effectiveness of different combinations of phages 2811, 311F, and 05F was compared using the virulence index scores to assess the efficacy (**Table 2**). The most effective doublet combination was 311F and 05F, which targets 53% of isolates. The addition of a further phage had a detrimental effect, reducing the number of isolates killed to 37% (**Table 2**).

Seven clinical isolates are targeted by the triplet cocktail compared to the six isolates covered based on the individual phage data (**Table 2**). The additional clinical isolate targeted by the triplet, KR398, showed a virulence index score (0.22) just above the threshold (**Table 2**). This suggests that for the *Klebsiella* phages, the killing seen with the individual phages translates directly to the triplet combination of phages. Additionally, the virulence index scores of the individual phages and of the triplet suggesting no synergy or competitive inhibition across all the clinical isolates. For example, clinical isolate KR438, phage 2811 only (0.78), triplet (0.73) or clinical isolate MH05 phage 05F only (0.32), triplet (0.33) (**Table 2**).

Analysis of the doublet (311F and 05F) showed unexpected synergistic combination. For five clinical isolates (KR358, KR359, KR360, KR396, and KR398), individually phages 311F and 05F

TABLE 2 | The virulence index scores of individual phage and various phage combinations across all the 19 ESBL-producing *Klebsiella* clinical isolates.

	2811	311F	05F	2811 311F	2811 05F	311F 05F	2811 311F 05F	Final
KR310	0.05	0.10	0.00	0.14	0.09	0.18	0.04	0.23
KR311	0.02	0.86	0.43	0.85	0.49	0.91	0.89	0.90
KR312	0.00	0.00	0.08	0.03	0.03	0.16	0.09	0.20
KR313	0.00	0.33	0.34	0.42	0.32	0.41	0.38	0.43
KR315	0.00	0.00	0.00	0.04	0.04	0.18	0.03	0.26
KR358	0.06	0.05	0.00	0.02	0.02	0.25	0.07	0.24
KR359	0.05	0.09	0.05	0.05	0.14	0.35	0.18	0.33
KR360	0.00	0.07	0.00	0.00	0.00	0.20	0.09	0.12
KR396	0.00	0.04	0.00	0.00	0.04	0.21	0.02	0.15
KR397	0.00	0.03	0.00	0.01	0.02	0.15	0.05	0.29
KR398	0.00	0.16	0.19	0.16	0.10	0.21	0.22	0.27
KR399	0.00	0.00	0.00	0.00	0.04	0.01	0.12	0.16
KR401	0.09	0.10	0.28	0.18	0.29	0.25	0.32	0.27
KR431	0.03	0.06	0.05	0.07	0.11	0.08	0.14	0.04
KR432	0.13	0.54	0.50	0.57	0.55	0.36	0.57	0.43
KR437	0.09	0.15	0.14	0.06	0.04	0.02	0.04	0.01
KR438	0.78	0.13	0.06	0.80	0.77	0.10	0.73	0.74
MH05	0.12	0.00	0.32	0.07	0.29	0.43	0.33	0.39
MH06	0.10	0.02	0.09	0.07	0.14	0.12	0.09	0.16
High > 0.5	1	2	1	3	2	1	3	2
Medium =0.2–0.5	0	1	4	1	4	9	4	11
Low > 0.001 – < 0.2	10	12	7	12	12	9	12	6
None 0	8	4	7	3	1	0	0	0
Effective	1/19	3/19	5/19	4/19	6/19	10/19	7/19	13/19
High (and Medium)	5%	16%	26%	21%	32%	53%	37%	68%

Numbers highlighted in Dark Blue represent high virulence index scores and those highlighted in Light Blue represent medium virulence index scores. The rows represent each of the ESBL Klebsiella clinical isolates used within this study. The top column represents the phage starting with individual phage on the left to progressing across the various combinations. The phages are highlighted with different colours: 2811 (Orange), 311F (Pink), 05F (Yellow), Final (Purple). Final = all six final phage (UP17, JK08, 113, 2811, 311F, and 05F). All values represent the mean generated from triplicate experimental data. A full diagrammatic representation of this data can be seen in ***Supplementary Figure S7****. The bottom section of the table summarises the individual phages and phage combinations into categories. The rows represent the categories, high = virulence index score > 0.5, medium = virulence index score 0.2–0.5, low = virulence index score ≥ 0.001–< 0.2, none = 0. Effective is a combination of the high and medium categories, this defines effective killing by the phage combination and its clinical isolate coverage.*

had an almost negligible effect, but when combined (311F and 05F) they demonstrated medium virulence index scores for all strains (**Table 2**). For the triplet cocktail (2811, 311F, and 05F), five clinical isolates (KR310, KR312, KR315, KR358, KR359, KR397) again had negligible virulence index scores (**Table 2**). But when exposed to the final cocktail (UP17, JK08, 113, 2811, 311F, and 05F), all five clinical isolates had a medium virulence index score (**Table 2**). This demonstrated a further unexpected synergy when added with the ESBL *E. coli* phages.

Effectiveness of the Final ESBL Phage Cocktail

The final ESBL cocktail was effective against 23/38 clinical isolates (61%) based on the virulence index data (any clinical isolates with a medium or high virulence index score > 0.2). The final ESBL cocktail was then tested in a 24 h biofilm assay, to test the cocktail in a bacterial virulence model.

The final ESBL cocktail was most effective against the *E. coli* clinical isolates. There was a significant ($p < 0.05$) decrease in bacterial cell viability in 11 (58%) and 13 (68%) isolates after 4 and 24 h of resazurin incubation, respectively (**Figure 3**).

For *Klebsiella*, at 4 h the cocktail only killed 5/19 (26%) of isolates and at 24 h 3/19 (16%) (**Figure 3**). This is in stark contrast to the high clinical isolate killing observed by the PKA of 13/19 (68%) (**Table 2**). An example of the disparity of results between the two tests is clinical isolate KR311. It had the highest virulence index score of 0.9 (**Table 2**), when using the final ESBL cocktail in the PKA but had no significant ($p < 0.05$) decrease in bacterial cell viability (**Figure 3**). However, the second highest virulence index score of 0.74 on isolate KR438 (**Table 2**) correlated with a significant ($p < 0.05$) reduction in the biofilm assay (**Figure 3**).

Genomic Analysis of the ESBL-Producing Clinical Isolates

Core genome SNP analysis was used to compare the clinical isolates (**Figure 4**). Ten different ST types of *E. coli* were identified, with the cocktail being able to target 10 strains across three ST types. The cocktail could target 5/6 of the ST131

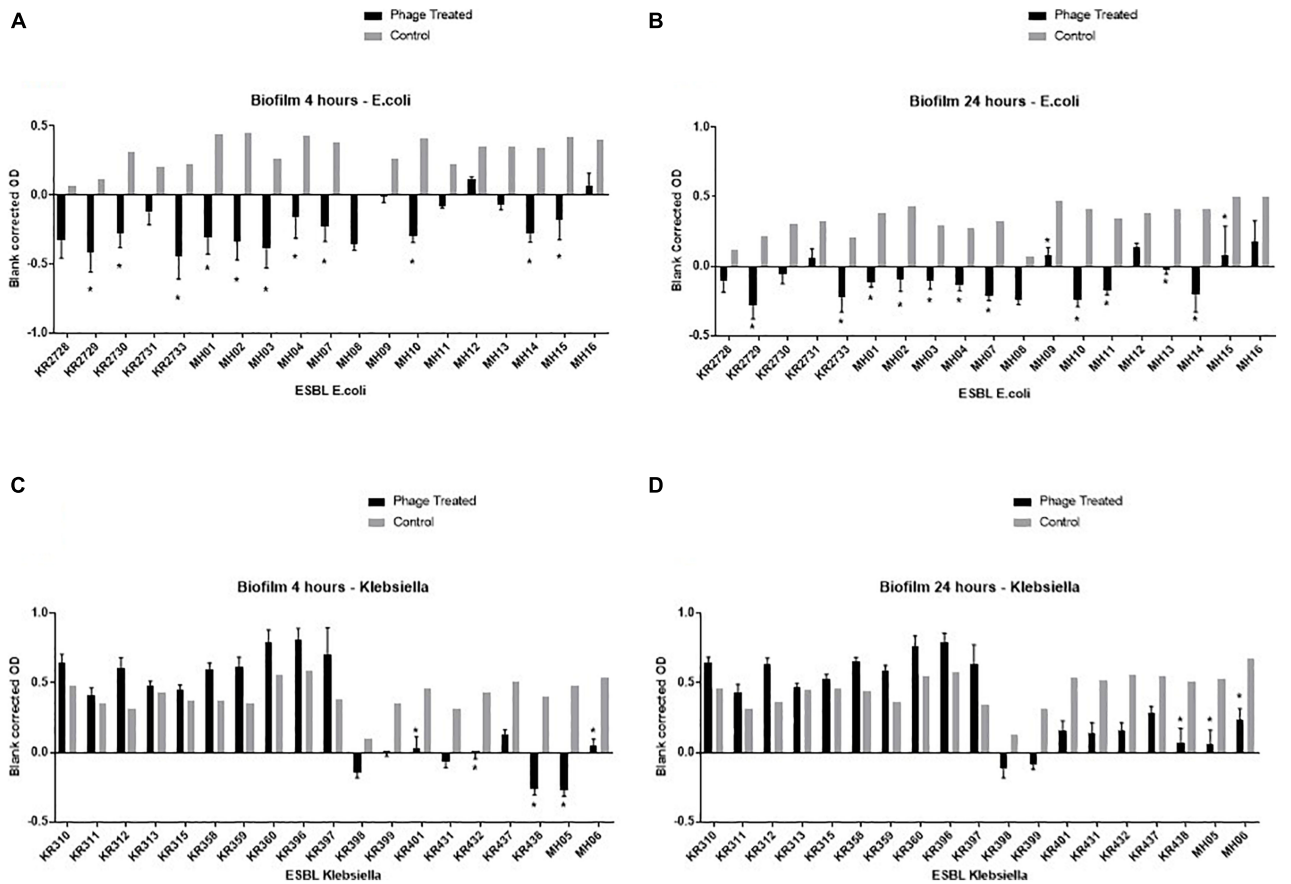

FIGURE 3 | Graphical representation of the biofilm assay data—resazurin cell viability-based model on a 96 well plate. Clinical isolates were grown for 24 h on a flat bottom 96 well plate, then incubated for an additional 24 h with either LB (control) or the final phage cocktail (phage treated). Resazurin was then added, OD readings were taken at 4 and 24 h post adding resazurin. Each ESBL-producing clinical isolate has two bars: the black bar represents the phage treated blank corrected OD and the grey bar represents the control blank corrected OD. OD taken at A_{595}, experiments repeated in triplicate for all clinical isolates, columns represents mean with standard error of the mean. * = significance difference between those treated with final phage cocktail and the control, $p < 0.05$. The top left graph **(A)** depicts the all ESBL producing *E.coli* clinical isolates 4 h post incubation with resazurin, top right **(B)** depicts ESBL producing *E.coli* clinical isolates 24 h post incubation with resazurin, the bottom left **(C)** depicts all ESBL-producing *Klebsiella* clinical isolates 4 h post incubation with resazurin and bottom right **(D)** depicts all ESBL-producing *Klebsiella* clinical isolates 24 h post incubation with resazurin.

clinical isolates, which is the most prevalent multidrug resistant uropathogen (Johnson et al., 2010; Kudinha et al., 2013). The core-genome SNP analysis of *Klebsiella* clearly separated the isolates into two different species (**Figure 4**). Three isolates were *Klebsiella oxytoca* and the remainder *Klebsiella pneumoniae.* There was a broad diversity of ST types present with 12 different types detected. There are representatives of the global endemic carbapenem-resistant associated ST258 as well as high risk AMR type ST147 (Bowers et al., 2015; Dhar et al., 2016; Peirano et al., 2020). The cocktail of phages was able to target a broad diversity of ST types across the three different bacterial species.

Figure 5 allows an overview of the PKA virulence index scores taking into consideration all phage combinations including individual, doublets, triplets and the six-phage final cocktail that were used during this work. It also includes combinations using phages that were screened but not selected as the final six phages.

When comparing this data with the two phylogenetic trees (**Figure 4**) of all 38 clinical isolates, there is no clear pattern of genomic similarities to phage susceptibility. The most sensitive *E. coli* clinical isolates (MH14, KR2733, KR2729, MH01, KR2728) are spread across three different clades. In contrast the most resistant clinical isolates were spread across five different clades (MH11, KR2731, MH12, MH13, and KR2730). With regards to *Klebsiella*, the most sensitive strains were spread across five clades (KR311, KR438, KR432, KR313, MH05). The most resistant strains were distributed across three different clades (KR399, KR396, KR360, KR315, KR397).

DISCUSSION

Antimicrobial resistance is an urgent issue that needs to be addressed. Phage therapy could be part of the solution. This work focuses on the development of an effective phage cocktail in response to this need. The aim of this work was to assess phage selection methods to streamline the development of a phage

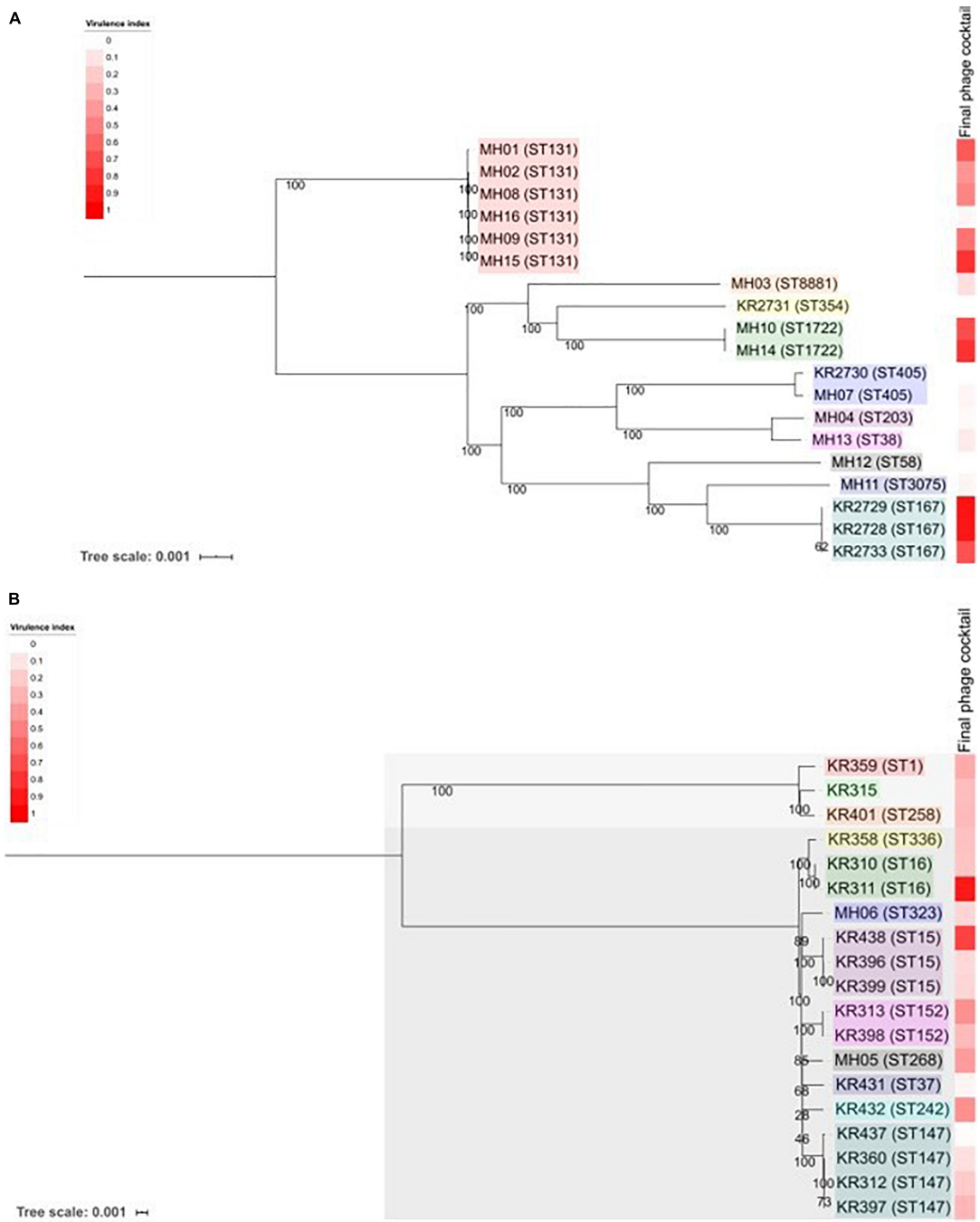

FIGURE 4 | Phylogenetic analysis of the 38 ESBL-producing clinical isolates used in this study. **(A)** Shows ESBL-producing *E. coli* and **(B)** shows ESBL-producing *Klebsiella* spp. Trees were produced using MEGA7 to assess the core genome SNPs. Core-genome SNP analysis revealed that there were two species of *Klebsiella*. KR315, KR359 and KR401 are *Klebsiella oxytoca* and all others are *Klebsiella pneumoniae*. Each clinical isolate name is followed by its MLST (Achtman—E. coli)—please note KR315 was unable to be assigned. Tree scale noted and bootstrap values are labelled on branches. Coloured boxes within each tree represent groups of sequence types. The heat map on each tree represents the virulence index score assigned to the final phage cocktail for each strain.

cocktail. This was achieved using an example of a phage cocktail against ESBL-producing clinical isolates of *E. coli* and *Klebsiella*.

DST is commonly used in the literature to assess the host range of phage (Sybesma et al., 2016; Hyman, 2019; Montso et al., 2019). The data demonstrated that the DST overestimates the host range or clinical isolate coverage of the individual phage by approximately 50% compared with the EOP and the PKA (**Figure 1**). The discrepancy between DST and EOP is in keeping with previous publications relating to Enterobacteriaceae species (Mirzaei and Nilsson, 2015; Manohar et al., 2019). It is considered

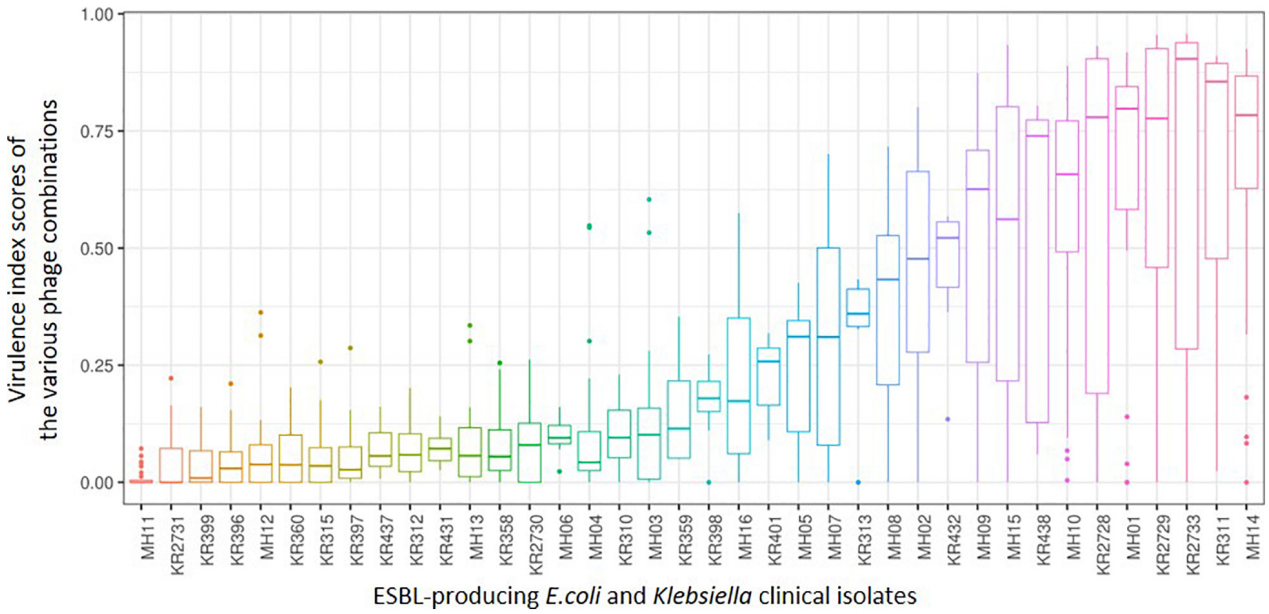

FIGURE 5 | A box and whisker plot depicting the range of killing assay virulence index scores from all phage combinations explored during this work for each of the 38 ESBL-producing clinical isolates. From left to right shows the ESBL-producing clinical isolates that are most resistant to the combinations attempted to those that are most sensitive. Please note not all combinations were completed in triplicate for those combinations that were not part of the final ESBL cocktail (all six phages).

to be due to other mechanisms of killing noted with DST, such as "lysis from without," that are not a result of phage replication (Hyman and Abedon, 2010). But when comparing EOP with PKA data, there is less disparity in the numbers of clinical isolates and specific individual clinical isolates covered. There is also a trade-off in experimental time between the methods described in terms of labour-intensity and actual experimental time under our laboratory conditions. For example, set-up time for DST ∼5 h, EOP ∼6 h versus PKA ∼2 h. In addition, EOP being the most labour intensive and requiring the most materials. This is compared to PKA which due to the 96-well plate format uses small volume of materials and allows the use of labour-saving device such as a multi-channel pipettor. So as EOP and PKA give similar results, the savings in labour and material would favour PKA.

Use of the virulence index score for analysis across a large dataset allowed direct comparison of individual phages and phage combinations, which would be a powerful tool for cocktail design. Overall, based on the dataset generated by this work, there is not a clear formula for the expected outcome when combining phages. This is due to either synergy or inhibition, which cannot be easily predicted. The ability of the virulence index to detect these interactions is a clear advantage over the use of DST or EOP as a selection method. The concepts of viral interference and augmentation have previously been discussed in the literature (Casey et al., 2018). Synergistic enhancement could be due to an effect on one or more of the three properties: (1) rate of infection, (2) production of progeny, or (3) the time window between infection and progeny release (Schmerer et al., 2014). Therefore, synergy of phage infection is an additional advantage for the creation of a successful phage cocktail (Schmerer et al., 2014). The PKA method alongside analysis using the virulence index score could make this a realistic research aim during future cocktail design. UP17 was interesting in that it also appeared to be resistant to interference from the other phages within the cocktail. This is shown with **Table 1**, where UP17 had a high virulence score against a particular clinical isolate (KR2728 and KR2729) this score is maintained throughout all the other phage combinations with UP17 (UP17 and JK08, UP17 and 113, UP17 and JK08 and 113, all 6).

Overall, it would be worth investigating further, why UP17 is resistant to interference from the other phages as well as to why its effectiveness increases when combined with JK08. In addition to this, also consider why JK08 and 113 had an antagonistic relationship. This could be due to the phages having similar receptor sites and one being more likely to lead to an abortive infection, or superinfection resulting in an unsuccessful infection for both (Abedon, 2015). Answering all of these questions, may help determine effective future cocktail design. The strength of this work is the use of the virulence index score to be able to support the combination of phages together in a cocktail by providing clear evidence of synergy. This synergy would not be apparent from other commonly used selection methods such as DST and EOP. In addition, this method also outperforms the previous PKA methods, with the use of time course measurements in a 96-well plate format, as it allows high throughput of a large number of individual phage/phage combinations and clinical isolate panels.

The data suggests that other factors may come into play for *Klebsiella* clinical isolates. When comparing the phage virulence assays of the biofilm assay and PKA, there appears to be no correlation for *Klebsiella* clinical isolates. For example, when

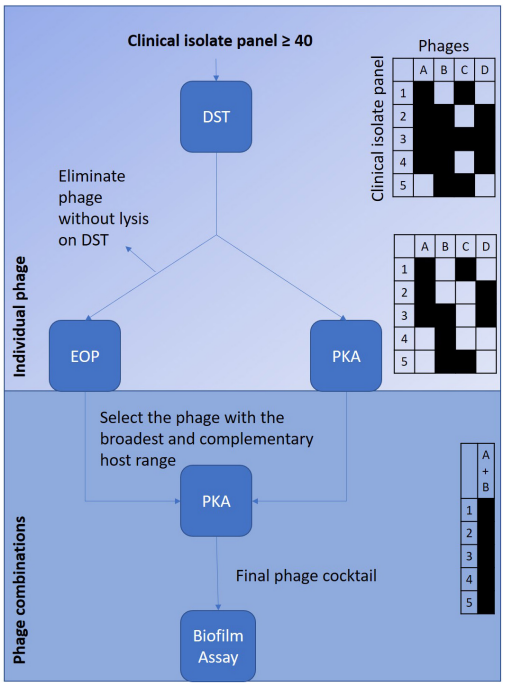

FIGURE 6 | Depicts the suggested selection processes to screen a phage library against a clinical isolate collection to optimise development of a potentially therapeutic phage cocktail. The left sided route suggests starting with the screening of the individual phages via the DST followed by EOP to confirm host-range. The right sided route suggests starting initially screening the individual phages with the DST followed by the PKA to confirm host-range. Both routes are then followed by selection of the phages that have the broadest and complementary host range to combine to test in combinations against the clinical isolate collection with the PKA. The final phage combination will be then be tested in biofilm assay. The tables on the right hand of the figure are to depict examples of the host-range coverage provided by each of the virulence tests.

using the final ESBL cocktail the clinical isolate KR311 has the highest virulence index score of 0.9 in the PKA (**Table 2**) and yet there was no significant ($p < 0.05$) decrease in the biofilm assay (**Figure 3**). In contrast, the second highest virulence index score of 0.74 against KR438 correlated with a significant ($p < 0.05$) reduction in cell viability in the biofilm assay (**Table 2** and **Figure 3**). Overall, when assessing the clinical isolates that demonstrated a significant ($p < 0.05$) reduction in cell viability for at least one of the timepoints during the biofilm assay, there appears to be no correlation with the PKA virulence index. This is a disappointing result, at least for this biofilm model, as the ideal case would be for the high-throughput method to determine virulence such as the PKA to translate to effectiveness in an *in vitro* bacterial model of virulence. The final cocktail covered 13 isolates (68%) in the PKA (**Table 2**) in comparison to 5/19 (26%) clinical isolates at 4 h and 3/19 (16%) clinical isolates at 24 h within the biofilm assay (**Figure 3**). It has been demonstrated in the literature that the use of phage can cause a significant reduction in biofilm production in *Klebsiella* spp. (Tabassum et al., 2018; Taha et al., 2018). This is in conflict to data demonstrated in this work. The results with *E. coli* were more promising as the clinical isolate coverage was similar 10/19 (53%) for PKA and in the biofilm assay 11/19 (58%) at 4 h and 13/19 (68%) at 24 h. The overall aim of this work was to develop a phage cocktail that was effective against 90% of the ESBL-producing clinical isolate collection. This was not achieved, and the result may influence the clinical application of the final cocktail. The data presented here demonstrated it was highly effective against the global prominent AMR UTI-associated *E. coli* ST131 isolates with 5/6 (83%) isolates killed. The data in this paper would need to be reconciled with prevalence data of the different sequence types within the general population to be able to draw conclusions with regards to the true clinical application.

In this study, we also performed basic bacterial genetic analysis identifying those clinical isolates that were most resistant to phage infection and those that were most sensitive. This highlighted clinical isolates that were on the same clade on the phylogenetic tree (**Figure 4**) but have polar opposite phage sensitivity. An example, *Klebsiella* KR396 and KR399 are both resistant isolates against sensitive isolate *Klebsiella* KR438 (**Figure 4**). Further genetic analysis of those with polar opposite phage sensitivity could provide further insight into mechanisms of resistance. It could also provide an opportunity to assess the individual clinical isolates susceptibility across three different screening methods and biofilm assay to see if there were any markers that predicted the outcome. These markers could help in the design of cocktails. In the future, a more detailed genomic analysis of the clinical isolates will be reported.

This paper is intended to outline the initial selection of phages for a final cocktail formulation. It will provide a basis for further building toward a "gold" standard within the community. This data could be used alongside other publications that have generated comparative data from the phage virulence methods described—DST, EOP, PKA and biofilm assay (Chen et al., 2018; Forti et al., 2018; Yang et al., 2020). There are two alternative routes that could be considered for use (**Figure 6**). Both commence with DST as the initial screening test. This would be used to eliminate phage candidates with low coverage of the clinical isolate collection. The refined list of phage candidates will either undergo EOP or PKA as individual phages against the clinical isolate collection to refine the list further. This will be achieved by providing a more accurate host range. In turn, this will allow selection of phages that have broad and complementary host ranges to ensure the widest coverage of the clinical isolate collection. Those phages selected will be used in various combinations within the PKA. When the optimal combination is elicited, it will be tested in the biofilm assay.

In conclusion, DST and EOP are not as useful as PKA as selection methods for designing phage cocktails. This is due to the inability of the DST and EOP to identify beneficial synergy as well as avoid inhibition. But DST and EOP are more easily accomplished and initially can add to the confidence of phage selection.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MH and MC designed the experiments. MH, JN, JM, DvS, JK, BA, and MA provided the phage used in the experiments. MH performed the DST, planktonic killing assays, and biofilm assays. MH and FH performed the EOP assays. JK, NB, and AT prepared phage genomic DNA for sequencing. FH and AM performed the bioinformatic analysis of the phage and bacterial genomes. MH, FH, DS, and NB analysed the data. MH interpreted the results. TS-P for figure design and creation. MH drafted the manuscript. FH, JN, JM, DS, AT, AM, EG, and MC edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.613529/full#supplementary-material

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