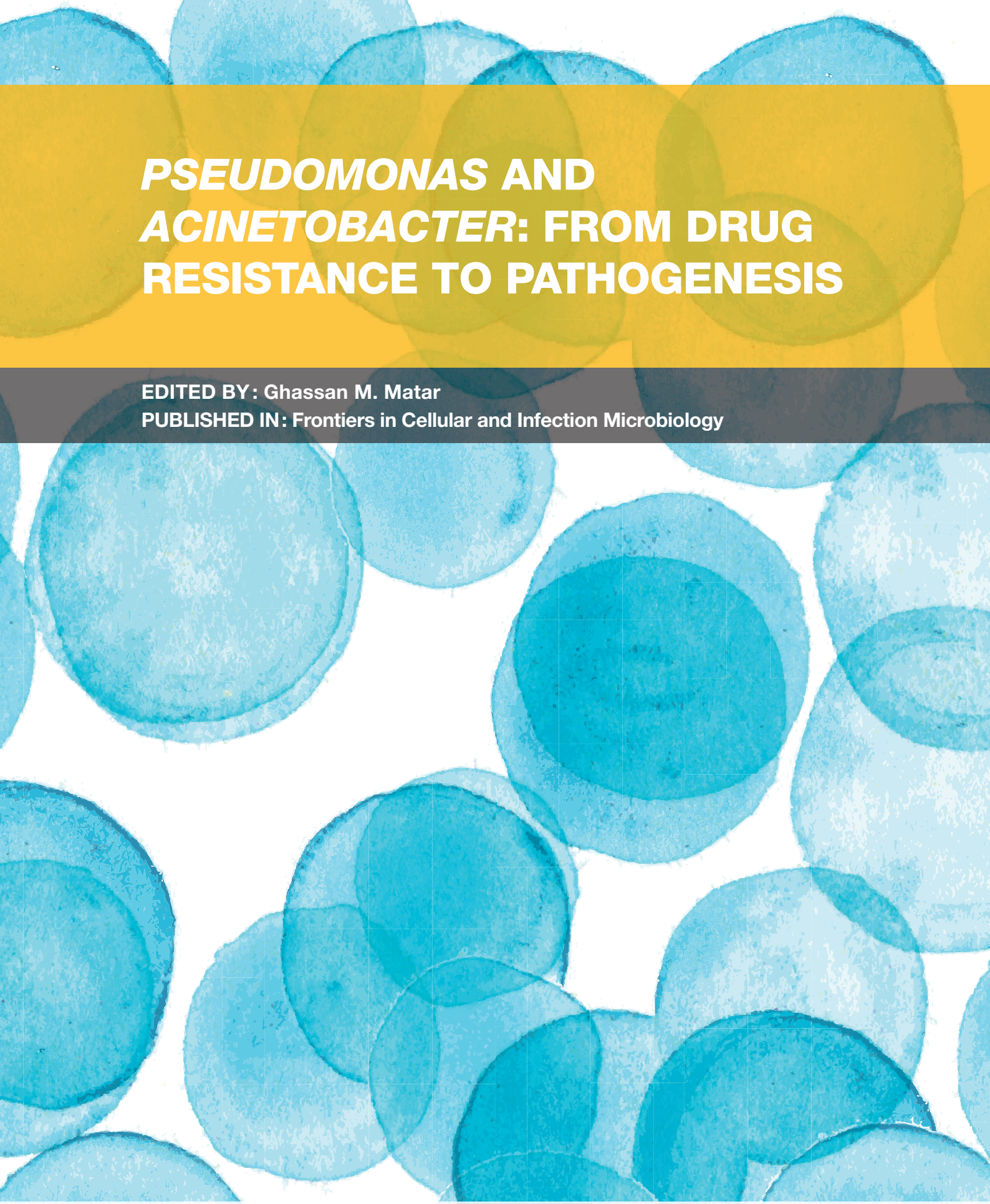
PSEUDOMONAS AND *ACINETOBACTER*: FROM DRUG RESISTANCE TO PATHOGENESIS

EDITED BY: Ghassan M. Matar

PUBLISHED IN: Frontiers in Cellular and Infection Microbiology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-469-3
DOI 10.3389/978-2-88945-469-3

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

PSEUDOMONAS AND *ACINETOBACTER*: FROM DRUG RESISTANCE TO PATHOGENESIS

Topic Editor:
Ghassan M. Matar, American University of Beirut, Lebanon

Pseudomonas aeruginosa and *Acinetobacter* baumannii are among the most common non-lactose-fermenting Gram-negative pathogens responsible for hospital-acquired infections, especially in intensive care units (ICUs). The treatment of infections caused by these bacteria is complicated due to the emergence of multi-drug resistance as the two species are noted for their intrinsic resistance to antimicrobial agents and their ability to acquire genetic elements that encode for resistance determinants. In both species, resistance to multiple classes of antimicrobial agents can seriously compromise the ability to treat infected patients, especially the immunocompromised. Consequently, very few antimicrobials remain as treatment options. Mechanisms of resistance in both of these pathogens include the production of β-lactamases and aminoglycoside-modifying enzymes as well as reduced or lack of expression of outer membrane proteins, mutations in topoisomerases, and up-regulation of efflux pumps.

To that purpose, the findings of the studies included in this book deal with the prevalence of resistant isolates to various antimicrobial agents in both *P. aeruginosa* and *A. baumannii*, their underlying mechanisms of resistance, their virulence factors, their pathogenesis, and prospective treatment options.

Special thanks are due to Mr. Bassam El-Hafi for facilitating procedures involved in this publication.

Citation: Matar, G. M., ed. (2018). *Pseudomonas* and *Acinetobacter*: From Drug Resistance to Pathogenesis. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-469-3

Table of Contents

IV. Antimicrobial Resistance:

V. Therapeutic Options:

frontiers in Cellular and Infection Microbiology

EDITORIAL
published: 13 March 2018
doi: 10.3389/fcimb.2018.00068

Editorial: *Pseudomonas* and *Acinetobacter*: From Drug Resistance to Pathogenesis

*Ghassan M. Matar**

Department of Experimental Pathology, Immunology and Microbiology, Center for Infectious Diseases Research, Faculty of Medicine, American University of Beirut, Beirut, Lebanon

Keywords: ***Pseudomonas***, ***Acinetobacter***, **pathogenesis, antimicrobial resistance, drug resistance, microbial**

Editorial on the Research Topic

***Pseudomonas* and *Acinetobacter*: From Drug Resistance to Pathogenesis**

The serious implications of *Pseudomonas* and *Acinetobacter* in healthcare setting outbreaks, along with their antimicrobial-resistant propensities, make it clear that these genera pose an imminent threat to public health. Both *Pseudomonas* and *Acinetobacter* employ numerous virulence factors that enhance their success as pathogens: biofilm formation, surface lipopolysaccharide, outer membrane proteins, secretion systems, etc. With respect to resistance, both microorganisms are intrinsically resistant to several classes of antibiotics. Furthermore, they can additionally acquire mobile genetic elements that confer further antimicrobial resistance; thus, pan-drug resistant isolates can be identified. Therefore, better understandings of the epidemiology, virulence, pathogenesis, and resistance patterns of and possible therapeutic options for these microorganisms are in constant demand.

When surveying *Acinetobacter*, Dahdouh et al. characterized 90 clinical isolates of *Acinetobacter baumannii* for their clonality, growth rates, antimicrobial susceptibility profiles, virulence factors, and mechanisms of carbapenem resistance. They discovered that most of their isolates belonged to international clone (IC) II, and had a high rate of carbapenem resistance; $bla_{\mathrm{OXA-23-like}}$ was the most common resistance gene. Despite these common features, there was no specific pattern of virulence, antimicrobial susceptibility profile, or growth rate associated with a clone. However, an association was found between surface motility, biofilm formation, and siderophore production. Taken together, these results imply that each *Acinetobacter* infection should be dealt with as a unique case, despite the presence of possible common clonality or antimicrobial resistance patterns. Additionally, a retrospective observational study by Ballouz et al. analyzed clinical outcomes and risk factors to predict mortality of patients with *Acinetobacter* bacteremia. It was observed that 91.1% of *A. baumannii* isolates causing bacteremia were extensively drug resistant (XDR). 75.3% of patients with *A. baumannii* bacteremia showed an Eastern Cooperative Oncology Group (ECOG) performance status of 4. Moreover, 70.37% of enrolled patient deaths were directly related to that bacteremia. No statistically significant differences in demographic, XDR profile, or source of bacteremia between survivors and non-survivors were identified; however, risk factors independently associated with mortality due to *A. baumannii* bacteremia included high-dose steroids and septic shock

OPEN ACCESS

Edited by:
D. Scott Merrell, Uniformed Services University, United States

Reviewed by:
Yousef Abu Kwaik, University of Louisville, United States

***Correspondence:**
Ghassan M. Matar
gmatar@aub.edu.lb

Received: *03 January 2018*
Accepted: *27 February 2018*
Published: *13 March 2018*

Citation:
Matar GM (2018) Editorial: Pseudomonas and Acinetobacter: From Drug Resistance to Pathogenesis. Front. Cell. Infect. Microbiol. 8:68. doi: 10.3389/fcimb.2018.00068

In terms of virulence factors, the review by Moradali et al. highlighted the clinical importance of *Pseudomonas aeruginosa* infections in cystic fibrosis (CF) patients and as a causative agent of hospital-acquired infections. Furthermore, the review described the most recent findings on adaption mechanisms employed by *P. aeruginosa* for survival; discussed topics included the quorum sensing (QS) system, biofilm formation, antimicrobial resistance mechanisms, adaptive radiation, stringent responses, and resistance to foreign DNA. In addition, a review by Hotterbeekx et al. discussed the interaction between *P. aeruginosa* and *Staphylococcus* spp. *in vitro* and *in vivo*. The presence of Gram-positive bacteria increases the production of extracellular virulence molecules by *P. aeruginosa*, which presumably serves as a defense mechanism. However, these molecules also damage host tissue, especially in the case of CF patients. On the other hand, *Staphylococcus aureus* adapts to *P. aeruginosa* by switching to the small colony variant (SCV) phenotype. This switch results in slowed cellular growth and cellular functions that result in increased survival in unfavorable conditions. In a multi-species biofilm, *S. aureus* can resist antibiotics by benefitting from the *P. aeruginosa* biofilm. As a result, the co-presence of *Staphylococcus* spp. and *P. aeruginosa* can worsen disease outcome.

On the other hand, the review by Lee et al. extensively characterized multiple biological aspects of *A. baumannii*, including virulence factors, antimicrobial resistance mechanisms, and possible treatment options. Pathogenic characteristics covered included porins, capsular and lipopolysaccharides, phospholipases, outer membrane vesicles, metal acquisition, biofilm formation, and protein secretion systems. Antimicrobial resistance mechanisms included efflux pump upregulation, β-lactamases of several classes, membrane permeability defects, and aminoglycoside-modifying enzymes. Possible treatment options encompassed carbapenems with β-lactamase inhibitors, tetracyclines, polymyxins, and phage therapy. Furthermore, Rumbo-Feal et al. analyzed the products of the gene cluster A1S_0112-A1S_0119 of *A. baumannii* ATCC 17978. They were able to verify that A1S_0112-A1S_0119 represents a polycistronic operon consisting of eight genes. Additionally, they determined that the A1S_0114 gene is associated with acetin 505 (Ac-505), which is needed for cell adherence. Three animal model studies showed that *A. baumannii* with an A1S_0114 gene deletion experienced significantly reduced virulence and cell adherence; thus, it was concluded that the A1S_0114 gene is involved in *A. baumannii* pathobiology.

When studying pathogenic behavior, López et al. investigated the effect of bile salts on the growth, virulence, and gene expression of *A. baumannii* clinical isolates and isogenic mutant strains that lack the AdeABC efflux pump. They reported that bile salts activated the *A. baumannii* QS system and modulated surface motility, biofilm formation, and the bacterial Type VI secretion system. In addition, bile salts also increased *A. baumanniii* invasiveness. As for *P. aeruginosa*, Bielen et al. made use of the chronic pneumonia agar bead model to examine innate immunity in relation to biofilm-like structures. They demonstrated that such a model in rats can activate the same anti-inflammatory type 2 (Th2) cytokines and innate immune cells that are also documented in CF patients; thus, these authors concluded that these cytokines can be used as biomarkers for infections in CF patients.

When studying resistance patterns, Hua et al. employed a multi-omics approach to examine colistin resistance mechanisms. Specifically, colistin resistance was induced in an *A. baumannii* clinical isolate and then whole genome sequencing (WGS), transcriptome and real-time quantitative PCR analysis, proteomics analysis, and growth rate studies were performed on the isolate. It was found that the growth rate of the mutant isolate was slower than that of the original strain. In addition, WGS showed the presence of ISAba1 upstream of *lpxC* in the mutant strain but not in the original isolate. Transcriptome and real-time quantitative PCR analysis revealed that 137 genes showed significant differential expression following induced resistance. Finally, proteomic analysis showed that while the expression of the AdeABC efflux pump was upregulated, certain biochemical pathways were downregulated in the mutant strain as compared to the original isolate.

Lu et al. explored the implications on conjugation of the interaction between the QS system of *P. aeruginosa* and the regulatory protein SdiA of *Escherichia coli*. They concluded that QS-SdiA interplay exerts an inhibitory effect on conjugation; the loss of *lasI* and *rhlI* genes in *P. aeruginosa* and the *sdiA* gene in *E. coli,* promoted the conjugation process. In addition, the authors concluded that repressed relaxase gene expression in donor cells might be the mechanism behind AHL inhibition of conjugation.

When identifying therapeutic options, Soudeiha et al. employed the checkerboard, time-kill curve, and perpendicular E-test assays to assess the effect of colistin and carbapenem combinations against MDR *A. baumannii* isolates that harbor several OXA-type carbapenemases. They found that while the checkerboard and perpendicular E-test assays show an additive effect for colistin and carbapenems, the time-kill curve assay showed a significant bactericidal effect for colistin and imipenem as compared to colistin and meropenem. In a different light, Du et al. demonstrated the bactericidal activity of the human salivary protein Histatin 5 (Hst 5) against ESKAPE pathogens, especially *P. aeruginosa* and *A. baumannii*. They concluded that Hst 5 exerts its bactericidal effect on *Enterococcus faecium* and *Enterobacter cloacae* upon internalization into the cell and on *P. aeruginosa* and *A. baumannii* through membrane disruption. The effect was delayed, but non-lytic, against *S. aureus*. Furthermore, Hst 5 was ineffective in killing *Klebsiella pneumoniae*. Their findings suggest the possibility of using Hst 5 as prophylactic topical antimicrobial therapy to prevent surgical, burn, and wound infections.

En masse, the articles included in this research topic help to shed light on the growing problems of drug resistance and pathogenesis of *Pseudomonas* and *Acinetobacter*.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

The author would like to acknowledge the work of Mr. Bassam El-Hafi for editing and compiling the information presented in this editorial.

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 25 November 2016
doi: 10.3389/fcimb.2016.00163

Acinetobacter baumannii Isolated from Lebanese Patients: Phenotypes and Genotypes of Resistance, Clonality, and Determinants of Pathogenicity

Elias Dahdouh[1], *Micheline Hajjar*[2], *Monica Suarez*[1] *and Ziad Daoud*[2]*

[1] Department of Animal Health, Faculty of Veterinary, Universidad Complutense de Madrid, Madrid, Spain, [2] Department of Clinical Microbiology, Faculty of Medicine, University of Balamand, Balamand, Lebanon

Introduction: *Acinetobacter baumannii* is a nosocomial pathogen that usually affects critically ill patients. High mortality rates have been associated with MDR *A. baumannii* infections. Carbapenem resistance among these isolates is increasing worldwide and is associated with certain International Clones (ICs) and oxacillinases (OXAs). Moreover, this organism possesses a wide range of virulence factors, whose expression is not yet fully understood. In this study, clinical *A. baumannii* isolates are characterized in terms of antibiotic resistance, mechanisms of carbapenem resistance, clonality, and virulence.

Materials and Methods: *A. baumannii* clinical isolates ($n = 90$) where obtained from a tertiary care center in Beirut, Lebanon. API 20NE strips in addition to the amplification of $bla_{OXA-51-like}$ were used for identification. Antibiotic susceptibility testing by disk diffusion was then performed in addition to PCRs for the detection of the most commonly disseminated carbapenemases. Clonality was determined by tri-locus PCR typing and doubling times were determined for isolates with varying susceptibility profiles. Biofilm formation, hemolysis, siderophore production, proteolytic activity, and surface motility was then determined for all the isolates. Statistical analysis was then performed for the determination of associations.

Results and Discussion: 81 (90%) of the isolates were resistant to carbapenems. These high rates are similar to other multi-center studies in the country suggesting the need of intervention on a national level. 74 (91.3%) of the carbapenem resistant isolates harbored $bla_{OXA-23-like}$ including two that also harbored $bla_{OXA-24-like}$. 88.9% of the *A. baumannii* isolates pertained to ICII and three other international clones were detected, showing the wide dissemination of clones into geographically distinct locations. Virulence profiles were highly diverse and no specific pattern was observed. Nevertheless, an association between motility, siderophore production, and biofilm formation was detected ($p < 0.05$).

Conclusions: A very high rate of carbapenem resistance was detected, showing the need for immediate intervention. IC II and OXA-23-like were the most disseminated,

OPEN ACCESS

Edited by:
Antoine Andremont,
Paris Diderot University, France

Reviewed by:
Bryan Troxell,
North Carolina State University, USA
Xuesong He,
University of California, Los Angeles School of Dentistry, USA

***Correspondence:**
Ziad Daoud
ziad.daoud@balamand.edu.lb

Received: *04 October 2016*
Accepted: *09 November 2016*
Published: *25 November 2016*

Citation:
Dahdouh E, Hajjar M, Suarez M and Daoud Z (2016) Acinetobacter baumannii Isolated from Lebanese Patients: Phenotypes and Genotypes of Resistance, Clonality, and Determinants of Pathogenicity. Front. Cell. Infect. Microbiol. 6:163. doi: 10.3389/fcimb.2016.00163

reflecting their international dissemination. No specific associations were made between virulence and resistance, but instead associations among certain virulence factors were found. Investigating a more clonally diverse pool of isolates could help in the determination of associations between virulence and resistance.

Keywords: *Acinetobacter baumannii*, carbapenem resistance, virulence, clonality, oxacillinases

INTRODUCTION

Acinetobacter baumannii is a nosocomial pathogen that could cause severe secondary infections among critically ill patients (Gordon and Wareham, 2010). This organism has a wide range of intrinsic resistance mechanisms and a heightened ability to acquire resistance to a broad range of antimicrobial agents (Peleg et al., 2012). Mortality rates among critically ill patients infected with Multi-Drug Resistant (MDR) *A. baumannii* are high, especially when improper empirical treatments are given (Ñamendys-Silva et al., 2015).

Carbapenems have been the treatment of choice for treating critically ill patients with MDR *A. baumannii* infections (Breilh et al., 2013). However, the increasing rates of Carbapenem Resistant *A. baumannii* (CRAB) isolates (Tärnberg et al., 2016) have limited their efficacy and increased mortality rates among infected patients (Lemos et al., 2014). Oxacillinases (OXAs) are the most commonly identified mechanism of carbapenem resistance among *A. baumannii* isolates. These OXAs include OXA-23-like, OXA-24-like, and OXA-58-like (Nowak and Paluchowska, 2016). *A. baumannii* also harbors the intrinsic $bla_{\mathrm{OXA-51-like}}$ in its genome. This OXA is highly conserved among *A. baumannii* strains (Turton et al., 2006a), but does not convey resistance to carbapenems unless associated with insertion sequences (Turton et al., 2006b). A few globally disseminated International Clones (ICs) have been found to cause most CRAB infections worldwide and have been associated with the presence of these OXAs (Karah et al., 2012).

A global surveillance program showed that Europe and the Mediterranean regions harbored the highest rate of MDR *A. baumannii* isolates (Flamm et al., 2016). Moreover, IC II was found to be widely disseminated among these countries (Di Popolo et al., 2011). This clone was also found to be largely disseminated in Lebanon (Rafei et al., 2014). Moreover, CRAB isolates were found to increase in prevalence in this country throughout the past decade (Hamouche and Sarkis, 2012). In 2012, 88% of 724 *A. baumannii* isolates recovered from various Lebanese hospitals were found to be resistant to imipenem (Hammoudi et al., 2015a). Although OXA-24-like (Hammoudi et al., 2015b) and OXA-58-like (Zarrilli et al., 2008) have been detected in this country, OXA-23-like seems to be the most prevalent among CRAB isolates (Rafei et al., 2015).

In addition to the various mechanisms of resistance detected among *A. baumannii* isolates, this organism can deferentially express various virulence factors. These factors include biofilm formation, surface motility, hemolysis on blood agars, siderophore production, and exoprotease activity; among others (Antunes et al., 2011). Whole genome sequencing and insertional mutagenesis led to the identifying of 28 genetic islands coding for genes predicted to be involved in virulence in this organism. Disruption of 6 of these islands did indeed result in avirulent isolates, as shown by infection models using *Caenorhabditis elegans* and *Dictyostelium discoideum* (Smith et al., 2007). Among the genes within these islands, genes coding for type IV secretion systems, which are associated with surface motility (Eijkelkamp et al., 2011a), are also found. Of note, *A. baumannii* was shown to produce different patterns of motility, depending on the choice (Difco Bacto or Eiken) or concentration of the agar (Clemmer et al., 2011). Also among the genes involved in virulence detected in *A. baumannii*, are genes coding for hemolysins/hemagglutinins (Smith et al., 2007). The expression of these genes was reported on blood agar plates and in liquid assays (Antunes et al., 2011). Exoprotease activity was also reported for *A. baumannii* strains in the previous study, after prediction of the presence of exoprotease genes in the *A. baumannii* genome (Antunes et al., 2011).

Siderophore production, mainly through the biosynthesis of acinetobactin, was also identified as a virulence determinant in *A. baumannii*. Successful biosynthesis of this siderophore was shown to be needed for the induction of apoptosis of epithelial cells. Moreover, impairment of siderophore production was shown to reduce the ability of *A. baumannii* to persist in and kill the host, as shown through infection models using *Galleria mellonella* larvae (Gaddy et al., 2012). Biofilm formation, though at different rates and patterns, has also been reported among *A. baumannii* clinical isolates (Dahdouh et al., 2016). Several loci in the *A. baumannii* genome have been implicated with the production of biofilms. One such locus is that coding poly-β-(1-6)-N-acetylglucosamine, an important component of biofilms, and a virulence factor that is involved in cell to cell adherence and protection from host defenses (Bentancor et al., 2012). Impairment of biofilm production, in addition to pili synthesis and motility, was shown to attenuate virulence in mammalian septicemia models (Cerqueira et al., 2014).

The relationship between virulence and antimicrobial resistance seems to be a highly complex one that is still not completely understood (Peleg et al., 2012). Importantly, the effect of harboring OXAs on virulence in *A. baumannii* is not well defined (Beceiro et al., 2013). The aim of this study is to characterize *A. baumannii* isolates obtained from a Lebanese hospital in terms of antibiotic susceptibility, carbapenemases harbored, clonality, and virulence determinants. Moreover, the relationship between virulence and carbapenem resistance will be explored. This would help in providing clinicians and infection control specialists with crucial data that would allow for the development of successful empirical treatments and infection control measures. Moreover, our findings could open the way for the exploitation of the

interplay between virulence and resistance in the clinical setting.

MATERIALS AND METHODS

Bacterial Strains

Ninety five non-repetitive *A. baumannii* clinical isolates were obtained from Saint George University—University Medical Center (SGH-UMC) over a period extending from June 2013 until August 2014. The isolates were obtained from various patient specimens that include sputum, pus, urine, and blood. The strains were identified using 20NE API strips (BioMérieux, France). The isolates that were not identified as *Acinetobacter calcoaceticus—A. baumannii* by the biochemical tests were not selected for further investigation. Amplification of the intrinsic $bla_{\text{OXA-51-like}}$ gene by PCR was then performed in order to verify that the isolate is *A. baumannii* (Turton et al., 2006a). The isolates were then stored at −20°C in Luria-Bertani (LB) Broth supplemented with 20% glycerol until used.

Antimicrobial Susceptibility Testing

The Kirby Bauer disk diffusion method was performed according to the CLSI guidelines (2014) in order to determine the Antibiotic Susceptibility Profiles (ASTs) of the isolates. The results were interpreted according to the cutoff values of the CLSI document M100-S24 (Clinical and Laboratory Standards Institute, 2014). Antimicrobial agents tested for were cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), piperacillin/tazobactam (100/10 μg), meropenem (10 μg), imipenem (10 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), ciprofloxacin (5 μg), gentamycin (10 μg), and colistin (10 μg). In order to verify resistance to colistin, *E*-tests (BioMérieux Mercy l'Etoile, France) and minimum inhibitory concentrations by broth microdilution were performed for isolates that showed narrow inhibition zones around the colistin disks.

Polymerase Chain Reactions

PCRs for the detection of $bla_{\text{OXA-51-like}}$ (Turton et al., 2006a); $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, and $bla_{\text{OXA-58-like}}$ (Mostachio et al., 2009); and $bla_{\text{OXA-48}}$, bla_{NDM}, and bla_{KPC} (Poirel et al., 2011) as previously described by the respective authors were performed. International lineage was determined using tri-locus PCR typing as described by Turton et al. (2007). The table summarized by Karah et al. (2012) was used in order to assign the international clonality of the tested isolates. For all PCR reactions, a commercial master mix was used (Qiagen, USA) and the primers, in addition to their respective annealing temperatures, are shown in **Table 1**. The PCR conditions were an initial elongation at 94°C for 3 min; followed by 30 cycles of 94°C for 45 s, the respective annealing temperature (**Table 1**) for 45 s, and 72°C for 1 min; and a final extension step at 72°C for 5 min. For the $bla_{\text{OXA-48}}$, bla_{NDM}, and bla_{KPC} genes, the number of cycles was increased to 36, and the annealing time was increased to 50 s (Poirel et al., 2011). Positive controls for the respective genes were used from previous studies (Moubareck et al., 2009; Hammoudi et al., 2015b).

Growth Curves

In order to calculate the doubling time for selected isolates with varying antibiotic susceptibility profiles, 1:100 dilutions from overnight cultures liquid cultures in fresh LB broth were performed. The fresh suspensions were then incubated at 37°C with shaking at 200 rpm for 8 h. Each hour, the OD_{600} was measured and the doubling times were calculated from the resulting curve as previously described (Hall et al., 2014). The growth curves and all the following experiments were performed in triplicates.

Biofilm Formation

Biofilm formation was detected in polystyrene tubes after staining with crystal violet as previously described (Tomaras et al., 2003). Briefly, 1 mL of inoculated LB broth was incubated overnight at 37°C and then washed and stained with 1% crystal violet for 10 min. The dye was then rinsed away and biofilms were visualized and graded as "++" if heavy stains were observed and as "+" if faint stains were observed.

Hemolytic Activity

Hemolytic activity on blood agar plates were tested for by inoculating 10 μL of a bacterial suspension adjusted to 10^6 CFU/mL in the center of these plates. The plates were then incubated for 6 days at 37°C and observed daily (Taybali et al., 2012).

Siderophore Production

Siderophore production in a liquid medium using the Chrome Azurol Solution (CAS) was performed as previously described (Louden et al., 2011). 5 mL of the PMS_7-Ca medium was inoculated and incubated for 72 h. The suspension was then centrifuged at 4000 × g for 10 min and 1 mL of the filter-sterilized supernatant was incubated 1:1 with the CAS. The OD_{630} was then measured and a 10% difference between the sample and un-inoculated PMS_7-Ca with CAS was considered as positive (Machuca and Milagres, 2003).

Surface Motility

For the detection of surface motility, a single colony was grown in LB broth overnight at 37°C. The visual turbidity of the suspension was then adjusted to be equivalent to the 0.5McFarland standard, in order to contain around 10^8 CFU/mL. 1 μL (10^5 CFU/mL) of the bacterial suspension was then inoculated on freshly prepared 0.3% LB-Agar (Difco, BD, USA) plates. The plates were then incubated at 37°C for 14 h and the diameter of the circular diffusion pattern was measured (Clemmer et al., 2011).

Proteolytic Activity

Proteolytic activity was determined by inoculating 5 mL of Trypticase Soy Broth Dyalisate with one colony and incubating it overnight at 37°C with shaking at 200 rpm. The following day, the suspension was centrifuged at 4000 × g for 10 min and 500 μL of the filter-sterilized supernatant was incubated with 500 μL of 1 mg/mL Azoalbumin dissolved in 50 mM Tris-HCl (pH = 7.7). This preparation was incubated at 37°C for 24 h and 13% trichloroacetic acid was then added. The tubes were incubated

TABLE 1 | Primers used in this study with their respective annealing temperatures.

Primer	Sequence	Annealing Temperature	References
Oxa-51-like-F	5′-ATGAACATTAAAGCACTC-3′	46°C	Turton et al., 2006b
Oxa-51-like-R	5′-CTATAAAATACCTAATTGTTC-3′		
Oxa-23-like-F	5′-GATCGGATTGGAGAACCAGA-3′	53°C	Mostachio et al., 2009
Oxa-23-like-R	5′-ATTTCTGACCGCATTTCCAT-3′		
Oxa-24-like-F	5′-GGTTAGTTGGCCCCCTTAAA-3′	53°C	Mostachio et al., 2009
Oxa-24-like-R	5′-AGTTGAGCGAAAAGGGGATT-3′		
Oxa-58-like-F	5′-AAGTATTGGGGCTTGTGCTG-3′	53°C	Mostachio et al., 2009
Oxa-58-like-R	5′-CCCCTCTGCGCTCTACATAC-3′		
KPC-F	5′-CGTCTAGTTCTGCTGTCTTG-3′	52°C	Poirel et al., 2011
KPC-R	5′-CTTGTCATCCTTGTTAGGCG-3′		
NDM-F	5′-GGTTTGGCGATCTGGTTTTC-3′	52°C	Poirel et al., 2011
NDM-R	5′-CGGAATGGCTCATCACGATC-3′		
Oxa-48-F	5′-GCGTGGTTAAGGATGAACAC-3′	52°C	Poirel et al., 2011
Oxa-48-R	5′-CATCAAGTTCAACCCAACCG-3′		
Group1ompAF306	5′-GATGGCGTAAATCGTGGTA-3′	57°C	Turton et al., 2007
Group1and2ompAR660	5′-CAACTTTAGCGATTTCTGG-3′		
Group1csuEF	5′-CTTTAGCAAACATGACCTACC-3′	57°C	Turton et al., 2007
Group1csuER	5′-TACACCCGGGTTAATCGT-3′		
Gp1OXA66F89	5′-GCGCTTCAAAATCTGATGTA-3′	57°C	Turton et al., 2007
Gp1OXA66R647	5′-GCGTATATTTTGTTTCCATTC-3′		
Group2ompAF378	5′-GACCTTTCTTATCACAACGA-3′	57°C	Turton et al., 2007
Group1and2ompAR660	5′-CAACTTTAGCGATTTCTGG-3′		
Group2csuEF	5′-GGCGAACATGACCTATTT-3′	57°C	Turton et al., 2007
Group2csuER	5′-CTTCATGGCTCGTTGGTT-3′		
Gp2OXA69F169	5′-CATCAAGGTCAAACTCAA-3′	57°C	Turton et al., 2007
Gp2OXA69R330	5′-TAGCCTTTTTTCCCCATC-3′		

at −20°C for 20 min and centrifuged at 15,000 × g for 10 min. The OD_{440} of the supernatant was then measured and U/L values were calculated where one U was the amount of enzyme needed to degrade one micromole of Azoalbumin (Ronca-Testoni, 1983; Antunes et al., 2011)

Statistical Analysis

Normality of the data, when applicable, was tested for using the Shapiro-Wilk test. One-way ANOVA and student *t*-tests were performed for normally distributed data while the Kruskal-Wallis and Mann-Whitney tests were performed non-normally distributed data. Qualitative data was analyzed using the chi squared and the two-sided Fisher's exact tests. *P*-values of less than 0.05 were considered as statistically significant and all tests were performed using the SPSS program, version 17.0 (SPSS 111 Inc., Chicago, USA).

RESULTS

Bacterial Isolates

In total, 95 *Acinetobacter* spp. isolates were collected. Of these isolates, three were *Acinetobacter haemolyticus, one* was *Acinetobacter junii/johnsonii*, and one was *Acinetobacter radioresistens/lwoffii* as identified by the API strips. The rest of the isolates ($n = 90$) were *A. baumannii* as identified by API and the subsequent amplification of $bla_{OXA-51-like}$ (Turton et al., 2006a). Thirty five (38.9%) *A. baumannii* isolates were collected in 2013 while fifty five (61.1%) were collected in 2014. Fifty eight (64.5%) of the *A. baumannii* isolates were collected from sputum, 17 (18.9%) from pus, 9 (10%) from urine, and 4 (4.4%) from blood samples. Two (2.2%) isolates were collected from catheters. Thirty six (40%) of the isolates were obtained from the ICU, 33 (36.7%) from general medicine, 10 (11.1%) from the surgical unit, 9 (10%) from the geriatric unit, 1 (1.1%) from the psychiatric unit, and 1 (1.1%) from the pediatric unit.

Antibiotic Susceptibility

Antibiotic susceptibility profiles were obtained after testing by the Kirby Bauer disk diffusion method according to CLSI guidelines (2014). Eighty one (90%) of the *A. baumannii* isolates were resistant to both meropenem and imipenem. Only one isolate was resistant to colistin. Susceptibility to other antimicrobial agents tested for was very low and did not exceed 14.4% (**Table 2**). Antibiotic susceptibility profiles are found in Supplementary Table 1.

Dissemination of Carbapenemases and International Clones

PCRs for the detection of the most common carbapenemases among *A. baumannii* were performed in addition to tri-locus

TABLE 2 | Antibiotic susceptibility profiles for 90 *A. baumannii* isolates collected over a 1-year period.

ANTIMICROBIAL AGENT	CTX		CAZ		FEP		TZP		MEM	
	n	%	n	%	n	%	n	%	n	%
Resistant	79	87.8	78	86.7	58	64.4	81	90	81	90
Intermediate	9	10	1	1.1	19	21.1	2	2.2	0	0
Sensitive	2	2.2	11	12.2	13	14.4	7	7.8	9	10
ANTIMICROBIAL AGENT	**IMP**		**SXT**		**CIP**		**GT**		**COL**	
	n	%	n	%	n	%	n	%	n	%
Resistant	81	90	83	92.2	84	93.3	77	85.6	1	1.1
Intermediate	0	0	0	0	0	0	0	0	0	0
Sensitive	9	10	7	7.8	6	6.7	13	14.4	89	98.9

CTX stands for cefotaxime, CAZ for ceftazidime, FEP for cefepime, TZP for piperacillin/tazobactam, MEM for meropenem, IMP for imipenem, SXT for trimethoprim/sulfamethoxazole, CIP for ciprofloxacin, GT for gentamycin, and COL for colistin.

PCR typing which determines international clonality. Of the 81 *A. baumannii* isolates that showed resistance to carbapenems, 74 (91.3%) harbored $bla_{\text{OXA-23-like}}$. Two of these isolates additionally harbored $bla_{\text{OXA-24-like}}$. These are isolates 42 and 49. None of the other carbapenemases tested for were detected. Seven isolates were resistant to carbapenems but did not harbor any of the tested carbapenemases (Isolates 54, 59, 68, 72, 83, 84, and 85). Two isolates were sensitive to carbapenems but harbored $bla_{\text{OXA-23-like}}$ (Isolates 17 and 87).

Eighty *A. baumannii* isolates (88.9%) pertained to IC II, whereas six (6.7%) pertained to group 4 (Isolates 11, 25, 39, 62, 82, and 91), one (1.1%) to group 10 (Isolate 56), and two (2.2%) to group 14 (Isolates 42 and 72), as summarized by Karah et al. (2012) (**Table 3**). One *A. baumannii* isolate showed a pattern that did not pertain to any IC where *CsuE* and $bla_{\text{OXA-66}}$ were amplified from the first multiplex, and the other allele of *CsuE* was also amplified from the second multiplex.

Virulence Determinants in Relation with Clonality and Carbapenem Susceptibility

The result of the various virulence determinants, in addition to international clonality and carbapenem resistance, are shown in **Table 3**. Seventy seven (85.6%) of the *A. baumannii* isolates showed strong biofilm formations while 10 (11.1%) showed weak formations and 3 (3.3%) showed no biofilm formation. Forty two (46.7%) of the *A. baumannii* isolates showed α-hemolysis on blood agar plates while one isolate showed β-hemolysis. Seventy two (80%) *A. baumannii* isolates showed a diffusion pattern indicating surface motility on 0.3% LB-Agar while 52 (57.8%) isolates were positive for siderophore production. Proteolytic activity ranged from 4.4 ± 1.63 U/L to 61.97 ± 12.65 U/L and the doubling times for selected isolates ranged from 0.262 ± 0.021 to 0.653 ± 0.049 h.

No general pattern was observed between the doubling times and antibiotic susceptibility profiles. The slowest doubling time was determined for isolate 28 which was carbapenem-resistant, α-hemolytic, produced strong biofilms, showed a motility pattern, harbored $bla_{\text{OXA-23-like}}$, and pertained to IC II. Nevertheless, other isolates with similar profiles showed faster doubling times (**Table 3**). One such example is isolate 78 which incidentally had the fastest doubling time. The colistin-resistant isolate 75 showed a similar antibiotic susceptibility pattern and virulence profile to the aforementioned two isolates and had a relatively fast doubling time of 0.328 ± 0.076 h.

Figure 1 shows representative isolates for non-motile, moderately motile, and highly motile isolates. Isolate 94 showed the highest motility diffusion diameter (144.5 ± 7.7 mm on a 15 cm square petri dish), produced strong biofilms, showed hemolysis on blood agars, and had a relatively high proteolytic activity (26.37 ± 4.42 U/L). This isolate was susceptible to carbapenems and pertained to IC II. Isolate 5 was the only isolate that showed β-hemolysis and it had the highest proteolytic activity. It also showed a motility diffusion pattern, produced strong biofilms and siderophores, pertained to IC II, and harbored $bla_{\text{OXA-23-like}}$. Isolates 2, 3, 4, 11, 12, and 82 showed very modest to no motility diffusion patterns and were negative for hemolysis. Isolate 4 also had a slow doubling time (0.594 ± 0.036 h). However, not all isolates that had similar motility diffusion diameters shared the rest of the virulence profile with these isolates.

Two of the isolates that pertained to group 4 were susceptible to carbapenems and four out of six isolates of this group were negative for siderophore production. Moreover, another two isolates of this group were negative for biofilm formation. Four of the six isolates pertaining to this group harbored $bla_{\text{OXA-23-like}}$ and were resistant to carbapenems. The isolate pertaining to group ten was susceptible to carbapenems and showed elevated levels of virulence determinants. Both isolates pertaining to Group 14 had similar profiles but one was negative for $bla_{\text{OXA-23-like}}$ but positive for hemolysis whereas the other harbored $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-24-like}}$ but was negative for hemolysis. Isolate 18, which did not pertain to any of the international clones by tri-locus sequence typing was susceptible

TABLE 3 | Virulence determinants of 90 *A. baumannii* isolates in addition to international clonality and carbapenem resistance.

Isolate	IC	Carb R.	Bio	Hemo	Sidero	Motility (mm)	Proteo (U/L)	DT (hours)
2	2	+	+	−	+	8 ± 0.7	7.66 ± 1.81	
3	2	+	++	−	+	7.5 ± 0.7	25.32 ± 4.22	
4	2	+	++	−	+	9.1 ± 0.5	28.9 ± 6.34	0.594 ± 0.036
5	2	+	++	β/D1	+	18.1 ± 2.8	61.97 ± 12.65	
7	2	+	++	α/D3	+	8.6 ± 1.2	23.68 ± 10.74	
8	2	+	+	α/D2	+	9.1 ± 1.1	20.56 ± 7.32	0.471 ± 0.067
10	2	+	++	α/D2	+	20.8 ± 3.3	5.59 ± 3.68	
11	4	−	++	−	+	6.4 ± 0.5	21.15 ± 2.06	
12	2	+	++	−	+	8.2 ± 1	3.33 ± 1.61	
13	2	+	+	α/D3	+	48.4 ± 2.6	7.47 ± 2.98	0.403 ± 0.056
14	2	+	++	α/D2	+	8 ± 0.9	26.22 ± 1.86	
15	2	+	++	−	−	8.2 ± 0.8	32.28 ± 5.44	0.539 ± 0.063
16	2	+	++	α/D3	+	14.2 ± 2	23.48 ± 1.96	
17	2	−	++	−	+	23.2 ± 1.4	16 ± 1.39	0.283 ± 0.031
18	-	−	++	−	+	25 ± 2.8	22.47 ± 3.39	
19	2	+	++	−	+	12.8 ± 0.6	23.24 ± 4.1	
20	2	+	++	α/D2	+	23.3 ± 1.7	30.69 ± 6.94	
21	2	+	++	−	+	43.1 ± 2.2	27.26 ± 0.63	0.377 ± 0.031
22	2	−	+	−	+	6.7 ± 0.8	5.98 ± 5.45	0.310 ± 0.063
23	2	+	++	α/D2	+	6.8 ± 0.7	24.71 ± 3.38	
24	2	+	++	−	+	16.9 ± 0.9	5.26 ± 1.42	
25	4	+	++	α/D2	+	26.1 ± 1.5	21.45 ± 3.9	
26	2	+	++	−	+	27.8 ± 1.5	8.72 ± 0.3	
27	2	+	++	−	+	22.9 ± 1.3	18.32 ± 0.63	
28	2	+	++	α/D2	−	27.5 ± 1.4	10.53 ± 4.43	0.653 ± 0.049
29	2	+	++	−	+	27.3 ± 1.5	32.27 ± 10.28	
30	2	+	++	−	+	18.8 ± 1	7.08 ± 3.31	
31	2	+	++	−	+	23.6 ± 2.1	27.71 ± 3.9	
32	2	+	++	−	−	30.3 ± 2.3	23.77 ± 1.77	
33	2	+	++	α/D2	+	37.7 ± 1.9	19.04 ± 2.15	
34	2	+	++	−	+	25.1 ± 1.5	23.24 ± 9.42	
35	2	+	++	−	+	24.4 ± 1.6	26.51 ± 1.86	
36	2	+	++	α/D2	+	41.5 ± 2.3	7.49 ± 4.58	
37	2	+	++	−	+	29.1 ± 2.4	23.24 ± 4.47	
38	2	+	++	−	+	25.6 ± 1.4	10.59 ± 3.46	
39	4	+	−	α/D3	−	10.3 ± 1.7	6.9 ± 2.4	
40	2	+	++	−	+	27.4 ± 1.9	6.68 ± 1.93	
41	2	+	++	α/D2	−	23.3 ± 1.5	9.09 ± 2.32	0.401 ± 0.030
42	14	+	++	−	+	23.2 ± 2.1	20.85 ± 4.92	
43	2	+	+	α/D3	+	30.6 ± 2.2	21.15 ± 7.6	
44	2	+	++	−	−	32 ± 2.4	19.48 ± 2.78	0.467 ± 0.039
45	2	+	++	−	+	28.9 ± 2.3	4.72 ± 2.17	
46	2	+	++	−	+	25.1 ± 1.5	11.05 ± 4.5	
47	2	+	++	α/D2	+	30.8 ± 2.3	15.94 ± 6.88	
48	2	+	++	α/D2	+	26 ± 1.6	11.65 ± 4.2	
49	2	+	++	−	+	19.2 ± 0.9	11.72 ± 4.99	0.343 ± 0.120
51	2	+	++	−	+	22.4 ± 1.5	24.43 ± 2.73	
52	2	+	++	α/D2	+	26 ± 2.2	9.43 ± 3.22	
53	2	+	++	α/D2	+	23.6 ± 2.4	11.4 ± 4.09	
54	2	+	++	−	−	20.3 ± 1.3	16.22 ± 5.1	
55	2	+	++	−	+	20.8 ± 1.3	30.47 ± 10	

(Continued)

TABLE 3 | Continued

Isolate	IC	Carb R.	Bio	Hemo	Sidero	Motility (mm)	Proteo (U/L)	DT (hours)
56	10	-	++	α/D3	+	31.5 ± 2.1	11.64 ± 2.02	
57	2	+	+	α/D2	−	21.8 ± 1.1	25.32 ± 2.73	
58	2	+	−	α/D1	−	11.1 ± 0.6	24.49 ± 4.55	
59	2	+	++	α/D2	−	22.1 ± 1.2	13.81 ± 4.49	
60	2	+	++	−	−	24.6 ± 1.8	8.37 ± 2.92	
61	2	+	++	α/D3	+	21.7 ± 1.1	21.61 ± 0.24	
62	4	−	++	α/D2	−	23.2 ± 1.4	7.3 ± 0.21	
63	2	+	++	α/D1	−	11.1 ± 0.7	4.4 ± 1.63	
64	2	−	+	α/D1	−	13.7 ± 0.6	10.6 ± 1.45	0.339 ± 0.065
65	2	+	++	α/D2	−	25.2 ± 1.9	7.38 ± 3.81	
66	2	+	++	α/D2	−	27.1 ± 2.2	27.41 ± 2.87	0.369 ± 0.021
67	2	+	++	α/D2	+	30.7 ± 1.9	22.94 ± 2.87	
68	2	+	++	−	−	30 ± 2.2	22.64 ± 7.44	
69	2	+	++	−	−	31.2 ± 2.8	28.3 ± 2.1	0.385 ± 0.056
70	2	+	++	α/D2	−	32.3 ± 1.7	22.05 ± 6.94	
71	2	+	++	−	−	28.3 ± 2	26.81 ± 3.1	
72	14	+	++	α/D3	+	20.2 ± 1.3	22.34 ± 2.36	
73	2	+	++	α/D1	−	34.7 ± 2.5	11.08 ± 5.56	
74	2	+	++	α/D2	−	28.3 ± 1.5	9.17 ± 0.93	
75	2	+	++	α/D2	−	22.3 ± 1.8	8.45 ± 3.1	0.328 ± 0.076
76	2	+	++	α/D3	+	24.6 ± 1.8	27.41 ± 4.03	0.311 ± 0.021
77	2	+	++	−	+	23.1 ± 5.9	12.02 ± 1.96	
78	2	+	++	−	−	35.7 ± 2	12.94 ± 2.61	0.262 ± 0.021
79	2	+	++	−	+	32.7 ± 1.8	11.13 ± 0.69	
80	2	+	+	α/D2	−	35.5 ± 1.8	22.94 ± 2.87	
81	2	+	++	α/D3	−	39.2 ± 1.5	26.81 ± 0.89	
82	4	+	−	−	−	11.1 ± 0.7	8.28 ± 0.33	
83	2	+	+	−	−	28.6 ± 2.4	20.85 ± 4.5	
84	2	+	++	−	−	28.9 ± 2.3	3.21 ± 3.04	
85	2	+	++	−	−	47.8 ± 2.6	8.49 ± 2.85	
86	2	+	++	−	−	24.6 ± 1.5	6.54 ± 4.7	
87	2	−	++	−	+	23.4 ± 1.9	13.9 ± 4.48	
89	2	+	++	−	−	41.5 ± 2.3	21.81 ± 8.09	
90	2	+	++	α/D2	−	47.9 ± 2.9	23.56 ± 10.31	
91	4	+	+	α/D3	−	16.2 ± 1	25.14 ± 2.31	
92	2	+	++	−	−	41.4 ± 2.1	6.04 ± 3.49	
93	2	+	++	α/D3	−	44.3 ± 2.5	24.55 ± 9.35	
94	2	−	++	α/D2	−	144.5 ± 7.7	26.37 ± 4.42	
95	2	+	++	−	−	46.6 ± 2.7	23.65 ± 8.61	0.414 ± 0.025

IC stands for international clone, Carb R. for carbapenem resistance, Bio for biofilm production, Hemo for hemolysis where the type of hemolysis and the day (D1–D3) on which it was first observed was recorded, Sidero for siderophore production, Proteo for proteolytic activity, and DT for doubling times.

to carbapenems and had similar virulence profiles as isolates pertaining to IC II. Both isolates that harbored $bla_{\mathrm{OXA-24-like}}$ in addition to $bla_{\mathrm{OXA-23-like}}$ were negative for hemolysis and had similar virulence profiles.

Associations between Virulence and Resistance

IC II was positively associated with both carbapenem resistance and harboring $bla_{\mathrm{OXA-23-like}}$ ($p < 0.01$). All the isolates that did not produce biofilms were also negative for siderophore production. The isolates that showed moderate motility diffusion patterns were associated with strong biofilm formation ($p < 0.01$) while those that were either highly motile or non-motile showed a positive association with siderophore production ($p < 0.05$). No other statistical association were made.

DISCUSSION

In this study, *A. baumannii* isolates obtained from a major tertiary care center in Beirut, Lebanon were characterized

FIGURE 1 | Representative isolates showing (from left to right) no motility, moderate motility, and high motility.

in terms of antibiotic susceptibility, clonality, and virulence determinants. An extremely high rate of carbapenem resistance (90%) was detected among the *A. baumannii* isolates. This rate, however, is very similar to that reported from a nation-wide study (Hammoudi et al., 2015a) where the prevalence of CRAB isolates was 88%. These findings suggest an immediate need for the implementation of effective infection control measures and antibiotic stewardship programs in Lebanese hospitals. This need is even more urgent due to the high resistance rates of these isolates to other antimicrobial agents that were tested for in this study (**Table 2**). The low rate of resistance to colistin among these isolates hold a viable alternative for treatment. Nevertheless, its nephrotoxic effects (Bergen et al., 2012) and the ability of *A. baumannii* to develop resistance toward this antimicrobial agent during therapy (Valencia et al., 2009) limits its effectiveness.

In accordance with other studies performed in Lebanon, IC II was by far the most prevalent clone among the *A. baumannii* isolates (Rafei et al., 2014) and OXA-23-like is the most disseminated (Rafei et al., 2015). These findings are also similar to those reported from other Mediterranean countries (Di Popolo et al., 2011). Interestingly, although an outbreak caused by $bla_{\text{OXA-58-like}}$ harboring CRAB isolates was reported from SGH-UMC a few years ago (Zarrilli et al., 2008), this carbapenemase was not detected among our isolates. This could be an indication of the successful eradication of the clone that caused the outbreak at the time of that study.

The presence of $bla_{\text{OXA-23-like}}$ in two carbapenem-sensitive isolates suggest that either the expression of this gene in these isolates is very modest or that it harbors a mutation that renders it ineffective. Further characterization of these isolates by sequencing the genetic environment of $bla_{\text{OXA-23-like}}$ and preforming RT-PCRs could help in better understanding why no carbapenem resistance was detected in these isolates. The seven CRAB isolates in which no carbapenemase was detected could be expressing resistance through carbapenemases that were not tested for in this study and/or through alteration of membrane permeability and efflux pump over-expression (Peleg et al., 2008).

Ten CRAB isolates pertaining to PCR group 4 were first identified in a study investigating *A. baumannii* isolates from several European countries (Towner et al., 2008). In our study, two out of the six isolates pertaining to this group were susceptible to carbapenems. Similarly, the isolate pertaining to group 10 was sensitive to carbapenems as opposed to the detection of carbapenem resistance among isolates pertaining to this group in a study in Portugal (Grosso et al., 2011). The two isolates pertaining to group 14 were both resistant to carbapenems. One of them did not harbor any of the tested carbapenemases while the other had both OXA-23-like and OXA-24-like. This group was first identified in a study from Romanian hospitals and the isolate pertaining to this group harbored $bla_{\text{OXA-58-like}}$ (Bonnin et al., 2011). The diversity of profiles between the isolates pertaining to these groups, in addition to the diversity seen among isolates pertaining to IC II, reflect the plasticity of the *A. baumannii* genome (Antunes et al., 2014). Moreover, the presence of these clones, in addition to the presence of the globally disseminated IC II (Karah et al., 2012), show the global expansion of *A. baumannii* clones that are able to expand to wide geographical areas.

A study by Antunes et al. (2011) showed that different *A. baumannii* clinical isolates are able to display different virulence profiles. This was indeed shown to be the case in our study where no specific pattern of virulence was associated with a specific clone (**Table 3**). The *A. baumannii* isolates investigated had a high degree of variability in terms of virulence profiles. Moreover, no associations between antibiotic susceptibility profiles and doubling times were detected. These findings suggest that each isolate should be treated as a unique case and no general assumptions could be made based on clonality and AST. This also shows that the relationship between virulence and antibiotic resistance is indeed a complex one and warrants further investigation (Peleg et al., 2012). Nevertheless, the low diversity of clones among the tested isolates could be obscuring associations between clonality and virulence, since some isolates pertaining to group 4 and those pertaining to group 14 had similar virulence profiles. Further investigating these associations using larger and more clonally diversified pools could shed further light on the matter. Interestingly, the motility diffusion patterns that were obtained in our study using Difco agar were circular, as opposed to the linear patterns reported while using this kind of agar by Clemmer et al. (2011). The patterns obtained in our study are rather similar to those obtained using Eiken agar. The reason for this difference is still not clear at this point, but could possibly be due to an experimental factor that is independent from the agar brand, or is a property pertaining to the particular strains that

were tested for in the different studies. This, however, requires further investigation before verification.

Finally, while comparing virulence factors one to another, an association between motility on one hand, and biofilm formation and siderophore production on the other, was determined. The relationship between motility and strong biofilm formation has been previously reported among MDR *A. baumannii* isolates (Eijkelkamp et al., 2011b). Moreover, the association detected between motility and siderophore production is not surprising since the former factor was associated with biofilm production while the latter allows for iron acquisition that is crucial for biofilm formation (Gentile et al., 2014). These associations reveal a highly complex interplay between the different virulence determinants in *A. baumannii*, especially those that are multi-factorial.

CONCLUSIONS

In conclusion, a very high rate of carbapenem resistance was detected among clinical *A. baumannii* isolates obtained from a Lebanese tertiary care center. IC II was the most prevalent clone and OXA-23-like was the most prevalent carbapenemase. The isolates showed highly varied virulence profiles that were not associated with any specific clone or oxacillinase gene. However, associations between motility, biofilm formation, and siderophore production have been found. Increasing the diversity of the pool of isolates could reveal associations between clonality and virulence that could allow for the prediction of pathogenicity of a clinical *A. baumannii* isolate.

AUTHOR CONTRIBUTIONS

ED: performed the virulence experiments, clonality analysis, part of the PCRs, and statistical analysis. Was also involved in experiment design and data analysis, and drafted the manuscript. MH: performed antibiotic susceptibility analysis and part of the PCRs, obtained the isolates that were included in this work, and was involve in the manuscript preparation. MS: was involved in study design, data analysis, and revision of the manuscript. ZD: was involved in study design, data analysis, revision of the manuscript, and supervised all the work that was done at his laboratory. All authors have approved the final version of the manuscript and are accountable for its content.

ACKNOWLEDGMENTS

Part of this work has been presented as a poster in the ECCMID conference in April, 2016. The authors would also like to acknowledge CNRS-Lebanon for partly funding the research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2016.00163/full#supplementary-material

REFERENCES

Antunes, L. C., Imperi, F., Carattoli, A., and Visca, P. (2011). Deciphering the multifactorial nature of *Acinetobacter baumannii* pathogenicity. *PLoS ONE* 6:e22674. doi: 10.1371/journal.pone.0022674

Antunes, L. C., Visca, P., and Towner, K. J. (2014). *Acinetobacter baumannii*: evolution of a global pathogen. *Pathog. Dis.* 71, 292–230. doi: 10.1111/2049-632X.12125

Beceiro, A., Tomás, M., and Bou, G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin. Microbiol. Rev.* 26, 185–230. doi: 10.1128/CMR.00059-12

Bentancor, L. V., O'Malley, J. M., Bozkurt-Guzel, C., Pier, G. B., and Maira-Litrán, T. (2012). Poly-N-acetyl-β-(1-6)-glucosamine is a target for protective immunity against *Acinetobacter baumannii* infections. *Infect. Immun.* 80, 651–656. doi: 10.1128/IAI.05653-11

Bergen, P. J., Landersdorfer, C. B., Zhang, J., Zhao, M., Lee, H. J., Nation, R. L., and Li, J. (2012). Pharmacokinetics and pharmacodynamics of 'old' polymyxins: what is new? *Diagn. Microbiol. Infect. Dis.*. 74, 213–223. doi: 10.1016/j.diagmicrobio.2012.07.010

Bonnin, R. A., Poirel, L., Licker, M., and Nordmann, P. (2011). Genetic diversity of carbapenem-hydrolysing β-lactamases in *Acinetobacter baumannii* from Romanian hospitals. *Clin. Microbiol. Infect.* 17, 1524–1528. doi: 10.1111/j.1469-0691.2011.03622.x

Breilh, D., Texier-Maugein, J., Allaouchiche, B., Saux, M. C., and Boselli, E. (2013). Carbapenems. *J. Chemother.* 25, 1–17. doi: 10.1179/1973947812Y.0000000032

Cerqueira, G. M., Kostoulias, X., Khoo, C., Aibinu, I., Qu, Y., Traven, A., et al. (2014). A global virulence regulator in *Acinetobacter baumannii* and its control of the phenylacetic acid catabolic pathway. *J. Infect. Dis.* 210, 46–55. doi: 10.1093/infdis/jiu024

Clemmer, K. M., Bonomo, R. A., and Rather, P. N. (2011). Genetic analysis of surface motility in *Acinetobacter baumannii*. *Microbiology* 157, 2534–2544. doi: 10.1099/mic.0.049791-0

Clinical and Laboratory Standards Institute (2014). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement*. M100-S24. Wayne, PA: Clinical and Laboratory Standards Institute.

Dahdouh, E., Orgaz, B., Gómez-Gil, R., Mingorance, J., Daoud, Z., Suarez, M., et al. (2016). Patterns of biofilm structure and formation kinetics among *Acinetobacter baumannii* clinical isolates with different antibiotic resistance profiles. *Med. Chem. Commun.* 7, 157–163. doi: 10.1039/C5MD00377F

Di Popolo, A., Giannouli, M., Triassi, M., Brisse, S., and Zarrilli, R. (2011). Molecular epidemiological investigation of multidrug-resistant *Acinetobacter baumannii* strains in four Mediterranean countries with a multilocus sequence typing scheme. *Clin. Microbiol. Infect.* 17, 197–201. doi: 10.1111/j.1469-0691.2010.03254.x

Eijkelkamp, B. A., Hassan, K. A., Paulsen, I. T., and Brown, M. H. (2011a). Investigation of the human pathogen *Acinetobacter baumannii* under iron limiting conditions. *BMC Genomics.* 12:126. doi: 10.1186/1471-2164-12-126

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Papadimitrious, M. S., Paulsen, I. T., and Brown, M. H. (2011b). Adherence and motility characteristics of clinical *Acinetobacter baumannii* isolates. *FEMS Microbiol. Lett.* 323, 44–51. doi: 10.1111/j.1574-6968.2011.02362.x

Flamm, R. K., Castanheira, M., Streit, J. M., and Jones, R. N. (2016). Minocycline activity tested against *Acinetobacter baumannii* complex, *Stenotrophomonas maltophilia*, and *Burkholderia cepacia* species complex isolates from a global surveillance program (2013). *Diagn. Microbiol. Infect. Dis.* 85, 352–355. doi: 10.1016/j.diagmicrobio.2016.03.019

Gaddy, J. A., Arivett, B. A., McConnell, M. J., López-Rojas, R., Pachón, J., and Actis, L. A. (2012). Role of acinetobactin-mediated iron acquisition functions in the interaction of *Acinetobacter baumannii* strain ATCC 19606T with human

lung epithelial cells, *Galleria mellonella* caterpillars, and mice. *Infect. Immun.* 80, 1015–1024. doi: 10.1128/IAI.06279-11

Gentile, V., Frangipani, E., Bonchi, C., Minandri, F., Runci, F., and Visca, P. (2014). Iron and *Acinetobacter baumannii* biofilm formation. *Pathogens* 3, 704–719. doi: 10.3390/pathogens3030704

Gordon, N. C., and Wareham, D. W. (2010). Multirdug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int. J. Antimicrob. Agents* 35, 219–226. doi: 10.1016/j.ijantimicag.2009.10.024

Grosso, F., Quinteira, S., and Peixe, L. (2011). Understanding the dynamics of imipenem-resistant *Acinetobacter baumannii* lineages within Portugal. *Clin. Microbiol. Infect.* 17, 1275–1279. doi: 10.1111/j.1469-0691.2011.03469.x

Hall, B. G., Acar, H., Nandipati, A., and Barlow, M. (2014). Growth rates made easy. *Mol. Biol. Evol.* 31, 232–238. doi: 10.1093/molbev/mst187

Hammoudi, D., Moubareck, C. A., Hakime, N., Houmani, M., Barakat, A., Najjar, Z., et al. (2015a). Spread of imipenem-resistant *Acinetobacter baumannii* co-expressing OXA-23 and GES-11 carbapenemases in Lebanon. *Int. J. Infect. Dis.* 36, 56–61. doi: 10.1016/j.ijid.2015.05.015

Hammoudi, D., Moubareck, C. A., Kanso, A., Nordmann, P., and Sarkis, D. K. (2015b). Surveillance of carbapenem non-susceptible gram negative strains and characterization of carbapenems of classes A, B and D in a Lebanese hospital. *Lebanese Med. J.* 63, 66–73. doi: 10.12816/0012553

Hamouche, E., and Sarkis, D. K. (2012). [Evolution of susceptibility to antibiotics of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumanii*, in a University Hospital Center of Beirut between 2005 and 2009]. *Pathol. Biol.* 60, e15–e20. doi: 10.1016/j.patbio.2011.03.011

Karah, N., Sundsfjord, A., Towner, K., and Samuelsen, Ø. (2012). Insights into the global molecular epidemiology of carbapenem non-susceptible clones of *Acinetobacter baumannii*. *Drug Resist. Updat.* 15, 237–247. doi: 10.1016/j.drup.2012.06.001

Lemos, E. V., de la Hoz, F. P., Einarson, T. R., McGhan, W. F., Quevedo, E., Castañeda, C., et al. (2014). Carbapenem resistance and mortality in patients with *Acinetobacter baumannii* infection: systematic review and meta-analysis. *Clin. Microbiol. Infect.* 20, 416–423. doi: 10.1111/1469-0691.12363

Louden, B. C., Haarmann, D., and Lynne, A. M. (2011). Use of blue agar CAS assay for siderophore detection. *J. Microbiol. Biol. Educ.* 12, 51–53. doi: 10.1128/jmbe.v12i1.249

Machuca, A., and Milagres, A. M. (2003). Use of CAS-agar plate modified to study the effect of different variables on the siderophore production by Aspergillus. *Lett. Appl. Microbiol.* 36, 177–181. doi: 10.1046/j.1472-765X.2003.01290.x

Mostachio, A. K., van der Heidjen, I., Rossi, F., Levin, A. S., and Costa, S. F. (2009). Multiplex PCR for rapid detection of genes encoding oxacillinases and metallo-beta-lactamases in carbapenem-resistant Acinetobacter spp. *J. Med. Microbiol.* 58, 1522–1524. doi: 10.1099/jmm.0.011080-0

Moubareck, C., Brémont, S., Conroy, M. C., Courvalin, P., and Lambert, T. (2009). GES-11, a novel integron-associated GES variant in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53, 3579–3581. doi: 10.1128/AAC.00072-09

Ñamendys-Silva, S. A., Correa-García, P., García-Guillén, F. J., González-Herrera, M. O., Pérez-Alonso, A., Texcocano-Becerra, J., et al. (2015). Outcomes of critically ill cancer patients with *Acinetobacter baumannii* infection. *World J. Crit. Care Med.* 4, 258–264. doi: 10.5492/wjccm.v4.i3.258

Nowak, P., and Paluchowska, P. (2016). *Acinetobacter baumannii*: biology and drug resistance - role of carbapenemases. *Folia Histochem. Cytobiol.* 54, 61–74. doi: 10.5603/FHC.a2016.0009

Peleg, A. Y., de Breij, A., Adams, M. D., Cerqueira, G. M., Mocali, S., Galardini, M., et al. (2012). The success of acinetobacter species; genetic, metabolic and virulence attributes. *PLoS ONE* 7:e46984. doi: 10.1371/journal.pone.0046984

Peleg, A. Y., Seifert, H., and Paterson, D. L. (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbiol. Rev.* 21, 538–582. doi: 10.1128/CMR.00058-07

Poirel, L., Walsh, T. R., Cuviller, V., and Nordmann, P. (2011). Multiplex PCR for detection of acquired carbapenemase genes. *Diagn. Microbiol. Infect. Dis.* 70, 119–123. doi: 10.1016/j.diagmicrobio.2010.12.002

Rafei, R., Dabboussi, F., Hamze, M., Eveillard, M., Lemarié, C., Gaultier, M. P., et al. (2014). Molecular analysis of *Acinetobacter baumannii* strains isolated in Lebanon using four different typing methods. *PLoS ONE* 9:e115969. doi: 10.1371/journal.pone.0115969

Rafei, R., Pailhoriés, H., Hamze, M., Eveillard, M., Mallat, H., Dabboussi, F., et al. (2015). Molecular epidemiology of *Acinetobacter baumannii* in different hospitals in Tripoli, Lebanon using bla(OXA-51-like) sequence based typing. *BMC Microbiol.* 15:103. doi: 10.1186/s12866-015-0441-5

Ronca-Testoni, S. (1983). Direct spectrophotometric assay for angiotensinconverting enzyme in serum. *Clin. Chem.* 29, 1093–1096.

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M., et al. (2007). New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev.* 21, 601–614. doi: 10.1101/gad.1510307

Tärnberg, M., Nilsson, L. E., and Dowzicky, M. J. (2016). Antimicrobial activity against a global collection of skin and skin structure pathogens: results from the Tigecycline Evaluation and Surveillance Trial (T.E.S.T.), 2010–2014. *Int. J. Infect. Dis.* 49, 141–148. doi: 10.1016/j.ijid.2016.06.016

Taybali, A. F., Nguyen, K. C., Shwed, P. S., Crosthwait, J., Coleman, G., and Seligy, V. L. (2012). Comparison of the virulence potential of Acinetobacter strains from clinical and environmental sources. *PLoS ONE* 7:e37024. doi: 10.1371/journal.pone.0037024

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., and Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology.* 149, 3473–3484. doi: 10.1099/mic.0.26541-0

Towner, K. J., Levi, K., Vlassiadi, M., and ARPA Steering Group (2008). Genetic diversity of carbapenem-resistant isolates of *Acinetobacter baumannii* in Europe. *Clin. Microbiol. Infect.* 14, 161–167. doi: 10.1111/j.1469-0691.2007.01911.x

Turton, J. F., Gabriel, S. N., Valderrey, C., Kaufmann, M. E., and Pitt, T. L. (2007). Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clin. Microbiol. Infect.* 13, 807–815. doi: 10.1111/j.1469-0691.2007.01759.x

Turton, J. F., Ward, M. E., Woodford, N., Kaufmann, M. E., Pike, R., Livermore, D. M., et al. (2006b).The role of ISAba1 in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. *FEMS Microbiol. Lett.* 258, 72–77. doi: 10.1111/j.1574-6968.2006.00195.x

Turton, J. F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E., and Pitt, T. L. (2006a). Identification of *Acinetobacter baumannii* by detection of the blaOXA-51-like carbapenemase gene intrinsic to this species. *J. Clin. Microbiol.* 44, 2974–2976. doi: 10.1128/JCM.01021-06

Valencia, R., Arroyo, L. A., Conde, M., Aldana, J. M., Torres, M. J., Fernández-Cuenca, F., et al. (2009). Nosocomial outbreak of infection with pan-drug resistant *Acinetobacter baumannii* in a tertiary care university hospital. *Infect. Control Hosp. Epidemiol.* 30, 257–263. doi: 10.1086/595977

Zarrilli, R., Vitale, D., Di Popolo, A., Bagattini, M., Daoud, Z., Khan, A. U., et al. (2008). A plasmid-borne blaOXA-58 gene confers imipenem resistance to *Acinetobacter baumannii* isolates from a Lebanese hospital. *Antimicrob. Agents Chemother.* 52, 4115–4120. doi: 10.1128/AAC.00366-08

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 04 May 2017
doi: 10.3389/fcimb.2017.00156

Risk Factors, Clinical Presentation, and Outcome of *Acinetobacter baumannii* Bacteremia

Tala Ballouz[1], Jad Aridi[1], Claude Afif[1], Jihad Irani[2], Chantal Lakis[3], Rakan Nasreddine[1] and Eid Azar[1]*

[1] Department of Infectious Diseases, Saint George Hospital University Medical Center and University of Balamand, Beirut, Lebanon, [2] Department of Family Medicine, Saint George Hospital University Medical Center and University of Balamand, Beirut, Lebanon, [3] Faculty of Medicine, University of Balamand, Beirut, Lebanon

Infections caused by *Acinetobacter baumannii* (AB), an increasingly prevalent nosocomial pathogen, have been associated with high morbidity and mortality. We conducted this study to analyze the clinical features, outcomes, and factors influencing the survival of patients with AB bacteremia. We retrospectively examined the medical records of all patients developing AB bacteremia during their hospital stay at a tertiary care hospital in Beirut between 2010 and 2015. Ninety episodes of AB bacteremia were documented in eighty-five patients. Univariate analysis showed that prior exposure to high dose steroids, diabetes mellitus, mechanical ventilation, prior use of colistin and tigecycline, presence of septic shock, and critical care unit stay were associated with a poor outcome. High dose steroids and presence of septic shock were significant on multivariate analysis. Crude mortality rate was 63.5%. 70.3% of the deaths were attributed to the bacteremia. On acquisition, 39 patients had septicemia. Despite high index of suspicion and initiation of colistin and/or tigecycline in 18/39 patients, a grim outcome could not be averted and 37 patients died within 2.16 days. Seven patients had transient benign bacteremia; three of which were treated with removal of the line. The remaining four did not receive any antibiotics due to withdrawal of care and died within 26.25 days of acquiring the bacteremia, with no signs of persistent infection on follow up. A prolonged hospital stay is frequently associated with loss of functionality, and steroid and antibiotic exposure. These factors seem to impact the mortality of AB bacteremia, a disease with high mortality rate and limited therapeutic options.

Keywords: *Acinetobacter baumannii*, extensive drug resistance, bacteremia, sepsis, risk factors, outcome

OPEN ACCESS

Edited by:
Lorenza Putignani, Bambino Gesù Ospedale Pediatrico (IRCCS), Italy

Reviewed by:
Ravi Jhaveri, University of North Carolina Hospitals, USA
Susu M. Zughaier, Emory University, USA

****Correspondence:***
Tala Ballouz
ballouztala26@gmail.com

Received: *30 January 2017*
Accepted: *12 April 2017*
Published: *04 May 2017*

Citation:
Ballouz T, Aridi J, Afif C, Irani J, Lakis C, Nasreddine R and Azar E (2017) Risk Factors, Clinical Presentation, and Outcome of Acinetobacter baumannii Bacteremia. Front. Cell. Infect. Microbiol. 7:156. doi: 10.3389/fcimb.2017.00156

INTRODUCTION

Acinetobacter baumannii (AB) is an aerobic non-fermenting gram-negative Coccobacillus, emerging as a prominent nosocomial pathogen with enhanced environmental resilience and propensity to develop resistance to commonly prescribed antimicrobials. Infections caused by AB include blood stream infections, ventilator associated pneumonias, urinary tract infections, meningitis, and wound infections (Munoz-Price and Weinstein, 2008). These infections are associated with high morbidity and mortality and contribute to a prolonged hospital stay and high hospital costs (Lee et al., 2007; Sunenshine et al., 2007; Jang et al., 2009; Asim et al., 2016).

Of particular importance is the ability of Acinetobacter to cause blood stream infections, especially in critically ill patients, the clinical course of which may range from a benign transient bacteremia to fulminant septic shock (Seifert et al., 1995).

Previous studies have demonstrated that crude mortality rates in patients with AB bacteremia varied between 30 and 76%, and factors associated with worse prognosis include immunosuppression (Gulen et al., 2015; Townsend et al., 2015; Gu et al., 2016), drug resistance (Lee et al., 2007; Sunenshine et al., 2007; Fu et al., 2015; Guo et al., 2016), severity of underlying illness (Seifert et al., 1995; Chopra et al., 2013, 2014; Nutman et al., 2014), inappropriate antimicrobial therapy (Esterly et al., 2011; Huang et al., 2012; Shorr et al., 2014; Freire et al., 2016), septicemia (Huang et al., 2012; Ñamendys-Silva et al., 2015; Freire et al., 2016), and prior antibiotic exposure (Chopra et al., 2013, 2014; Gu et al., 2016; Liu et al., 2016).

The epidemic of AB at our institution began in 2010 as an outbreak in the intensive care unit (ICU), rapidly evolving into an endemic with high level resistance, most likely secondary to selective pressure caused by heavy carbapanem usage. Despite implementation of infection control practices and antibiotic restriction, the incidence of AB bacteremias continued to increase (**Figure 1**). So we conducted this study to analyze the clinical outcomes and risk factors predicting mortality in patients with *Acinetobacter bacteremia*.

MATERIALS AND METHODS

Study Design

For this purpose, we designed and conducted a retrospective observational study at Saint George Hospital University Medical Center (SGHUMC), a 400 bed tertiary medical center in Beirut, Lebanon. We reviewed the medical records of patients who were admitted between June 2010 and March 2015 and developed *Acinetobacter baumannii* (AB) bacteremia.

Data Collection

An extensive data collection sheet was designed and looked at demographic factors such as age, gender, dates of admission and discharge, admission diagnosis, functional status at admission, and at acquisition of AB. We also gathered data on medical comorbidities, critical care unit stay, invasive procedures prior to acquisition (PTA) of bacteremia, antibiotic and steroid exposure, prior AB infection, sources and clinical manifestation of bacteremia, antimicrobial susceptibility, treatment (time of initiation, doses, routes), and outcome.

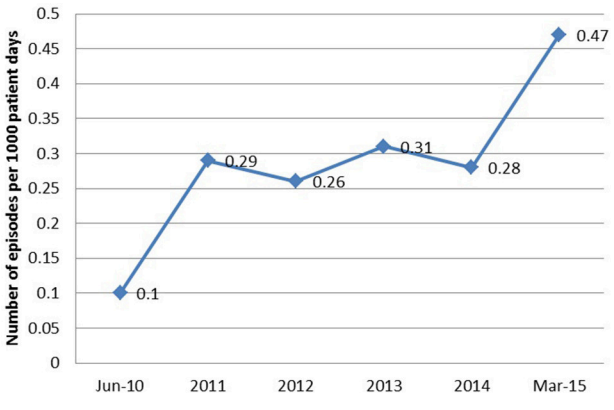


FIGURE 1 | The annual incidence of *Acinetobacter baumannii* bacteremia from June 2010 to March 2015.

Organism Identification and Resistance Profile Classification

At patient bedside, the blood samples were collected directly into BACTEC® culture vials (Becton Dickinson, Heidelberg, Germany). Antimicrobial susceptibility was performed by disc diffusion test and results were interpreted according to Clinical Laboratory Standards Institute Criteria. Extensive drug resistance (XDR) was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial classes.

Definitions

Functionality of the patients was obtained according to the Eastern Cooperative Oncology Group (ECOG) performance status: (0) Fully active, able to carry on all pre-disease performance without restriction, (1) Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work, (2) Ambulatory and capable of all self-care but unable to carry out any work activities; up and about more than 50% of waking hours (3) Capable of only limited self-care; confined to bed or chair more than 50% of waking hours (4) Completely disabled; cannot carry on any self-care; totally confined to bed or chair, (5) Dead.

High dose steroid was noted to be any dose of steroid of $\geq$1 mg/kg/day equivalent of prednisone. Previous antibiotic therapy was defined as receiving any systemic antibiotic prior to the positive blood culture for more than 48 h. A critical care stay was noted when a patient had a stay at the Intensive Care Unit (ICU), the Cardiac Care Unit (CCU), or the Cardiothoracic Surgery Unit (CSU) for more than 24 h. Neutropenia was defined as an absolute neutrophil count $<$1,500 cells/mm^3. Antimicrobial treatment was defined as "active" if the antibiotics which were administered within 24 h of bacteremia onset, included at least one antibiotic that was active *in vitro*. Bacteremias were classified as primary and secondary. A bacteremia was considered to be secondary when the source is known (pulmonary, gastrointestinal, urinary, wound). A primary bacteremia was either line related or when no apparent source was evident and it was assumed that the patient acquired AB bloodstream infection through colonization of their skin. Septic shock was defined as persistent hypotension despite fluid replacement, requiring vasopressors. We chose in-hospital mortality as the primary outcome measure. A panel of two infectious diseases physicians, two clinical fellows, and a research fellow reviewed the patients' medical records and causes of death were divided into unrelated and related. Unrelated was defined as death that was completely separate from the bacteremia event, related death was defined as death directly due to the AB bacteremia event.

Statistical Analyses

Continuous variables were presented by mean values ± standard deviation and interquartile ranges while categorical ones were presented by percentages. Categorical variables were analyzed using Pearson's chi-square test and continuous variables were analyzed using Student's *t*-test or Mann–Whitney test. To determine independent risk factors for mortality, a multiple logistic regression analysis was used to control for the effect of confounding variables. The results of this regression analyses were reported as adjusted odds ratio (OR) with 95% confidence interval (CI). Time to mortality defined as length of stay post acquisition of bacteremia, in related and unrelated death was analyzed using Kaplan Meier survival analysis, and the log rank test was used for comparison between the two groups. A $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software version 20.0.

RESULTS

From June 2010 to March 2015, a total of 90 episodes of AB bacteremias were documented with an increasing incidence from 0.1 per 1,000 patient days in 2010 to 0.47 in 2015 (**Figure 1**). This was associated with an increase in the resistance profile of AB, where in 2010 all isolates were susceptible, but by 2015 all isolates became XDR (**Figure 2**).

Antimicrobial Susceptibility

We found that the majority of our isolates were carbapenem resistant (82/90). Seventy-nine isolates (87.7%) were deemed to be XDR, as defined in the Methods Section. Tigecycline had the highest level of *in-vitro* sensitivity at 83.3%. The isolates were highly resistant to piperacillin/tazobactam (87/90), ciprofloxacin (86/90), ceftazidime (85/90), cefepime (83/90), trimethoprim/sulfamethoxazole (82/90), and amikacin (81/90). All isolates were sensitive to colistin by disc diffusion. Moreover, colistin susceptibility by broth microdilution was done in five isolates and all were found to be susceptible.

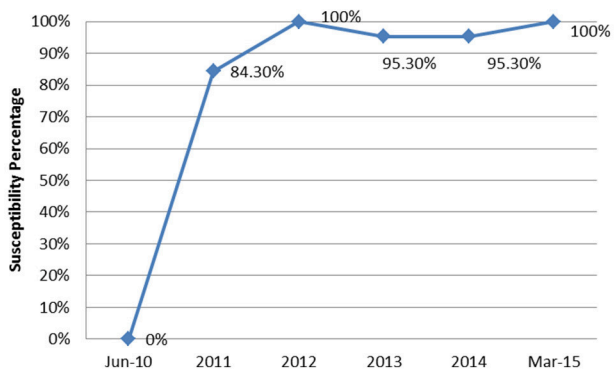


FIGURE 2 | The annual trend of the extensively drug resistant rate of *Acinetobacter baumannii* isolates from June 2010 to March 2015.

Patient Characteristics

Demographics and baseline characteristics of the study population are shown in **Table 1**. Mean age of the patients was 67.14 ± 20.34 years (range 8 days–92 years). The bacteremia was mostly prevalent (54.5%) in the 70–90 years age group. 62.3% of the study population was male.

On admission, 24 patients (28.2%) had an ECOG of 3, being confined to bed or chair for more than half of the day. Thirty-seven patients (43.5%) had an ECOG of 4, with inability to carry any daily life activities. On acquisition of the bacteremia, 75% of the patients had an ECOG of 4.

Seventy-one patients (83.5%) had at least one comorbidity, hypertension being the most prevalent (67%). Thirty-eight patients (44.6%) had a malignancy, half of which were hematological.

Patients spent a mean duration of 18.97± 16.79 days in the hospital before developing AB bacteremia.

Seventy-seven (90.5%) patients had at least one invasive procedure PTA, most commonly foley and central catheters.

67.7% of the episodes in 58 patients were preceded by a critical care (CC) unit stay. Mean time interval from CC unit admission to developing bacteremia was 9.94 ± 8.32 days.

Twenty-five patients had antibiotic exposure prior to their admission to the hospital and 93.3% of the events were preceded by exposure to at least one antimicrobial (**Table 2**). Prior exposure to colistin and/or tigecycline was documented in 25 events; 15 of which were for a prior AB infection. Otherwise, they were given empirically.

Fifty patients received high dose steroids, most commonly for sepsis, for a mean duration of 8.4 ± 11.21 days. The indications for steroids are listed in **Table 3**.

Event Characteristics and Outcome

Clinical characteristics of bacteremia events are listed in **Table 4**.

Fifty-seven (63.3%) bacteremia events were considered primary, and thirty-three (36.7%) were secondary to a source of infections, lungs being the most common. Septic shock was present at the time of acquisition in 41 events (45.5%).

Overall, 54 patients (63.5%) died during their hospitalization with a mean duration of infection to death of 16.03 ±20.31 days. Thirty-eight patients died from causes directly related to the bacteremia, while 16 died from unrelated causes. **Figure 3** shows the duration of survival which was significantly shorter in patients who died from related causes (2.16 ± 3.36 vs. 25.25 ± 15.25, $p < 0.001$).

We described three outcomes after acquisition of the bacteremia. The first was where patients recovered without administration of antibiotics (seven patients); three of them were effectively treated by removal of the offending line. The remaining four patients had documented bacteremia and did not receive any treatment; they died after a mean duration of 26.25 days after withdrawal of their care as by the family's wishes. All seven patients had no evidence of persistent signs of infection as by their daily follow up. The second group included 39 patients, with fulminant sepsis on acquisition. Thirty-seven or Thirty-nine died and the time to death was as short as 2.1 days even in those who received active treatment (17/37).

TABLE 1 | Demographics, clinical characteristics, and exposure prior to *Acinetobacter baumannii* bacteremia event[a].

Variables	Number
Age, mean (SD)	67.14 ±20.34
Sex (%)	
Male	53 (62.3)
Female	32 (37.6)
Comorbidities (%)	
Hypertension	57 (67)
Malignancies	38 (44.6)
Hematologic	19 (22.3)
Acute leukemia	8 (9.5)
Chronic lymphocytic leukemia	2 (2.3)
Lymphoma	5 (5.9)
Myelodysplastic syndrome	2 (2.3)
Multiple myeloma	2 (2.3)
Solid	19 (22.3)
Diabetes mellitus	25 (29.4)
Coronary artery disease	25 (29.4)
Congestive heart failure	10 (11.7)
Renal disease	15 (17.6)
Dyslipidemia	11 (12.9)
Autoimmune diseases	6 (7)
Lung disease	18 (21.2)
Cerebral vascular accident	6 (7)
Neutropenia prior to event[b] (%)	13 (15.3)
ECOG (on admission) (%)	
1	7 (8.2)
2	14 (16.5)
3	24 (28.2)
4	37 (43.6)
N/A	3 (3.5)
ECOG (on acquisition) (%)	
1	3 (3.5)
2	5 (5.9)
3	10 (11.8)
4	64 (75.3)
N/A	3 (3.5)
Invasive procedures prior to event[b] (%)	
Foley	68 (76.4)
Central lines	68 (76.4)
Nasogastric tube	40 (44.9)
Mechanical ventilation	40 (44.9)
Drains	19 (21.1)
Tracheostomy	7 (7.9)
TPN (%)	16 (17.8)
Antibiotic exposure (%)	
Prior to admission	25 (27.8)
Prior to event[b]	84 (93.3)
High dose steroid exposure prior to event[b]	53 (58.9)
CC unit stay prior to event[b]	60 (66.7)
LOS prior to event, mean, days[b]	18.97 ±16.79

SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; TPN, total parenteral nutrition; CC, critical care; LOS, length of stay.

[a] *Data are presented as n (%), unless otherwise specified.*

[b] *Variables are presented by episodes (n = 90), otherwise they are presented by patients (n = 85).*

TABLE 2 | Antibiotic exposure prior to *Acinetobacter baumannii* bacteremia event (*N* = 90).

Antibiotic	Number (%)
Quinolones	32 (35.5)
Antipseudomonal cephalosporins (cefepime, ceftazidime)	15 (16.7)
Carbapenems (meropenem, imipenem)	59 (65.6)
Colistin	17 (18.8)
Tigecycline	16 (17.8)
Other beta lactams (piperacillin/tazobactam, amoxicillin, ceftriaxone)	31 (34.4)
Aminoglycosides	7 (7.8)
Metronidazole	35 (38.9)
Glycopeptides	38 (42.2)

TABLE 3 | Indications for high dose steroid exposure (*N* = 53).

Indication	Number (%)
Sepsis	15 (28.4)
Bronchospasm	8 (15.1)
Chemotherapy	8 (15.1)
ARDS	4 (7.5)
Immune (MAS, SLE)	6 (11.3)
More than one indication	8 (15.1)
Other	4 (7.5)

ARDS, acute respiratory distress syndrome; MAS, macrophage activating syndrome; SLE, systemic lupus erythematosis.

TABLE 4 | Clinical characteristics of the *Acinetobacter baumannii* bacteremia events (*N* = 90).

Variables	Number (%)
Source	
Primary	57 (63.3)
Secondary	
Lungs	20 (22.2)
Urine	3 (3.3)
Intraabdominal	5 (5.6)
Wound/Soft tissue	5 (5.6)
Resistance profile	
XDR	82 (91.1)
Non-XDR	8 (8.9)
Septic shock on acquisition	41 (45.6)
Active treatment	
Within 24 h of positive blood culture results	32 (35.6)

XDR, extensively drug resistant.

The remaining 41 patients recovered from the bacteremia after receiving appropriate antibiotics. Of those, 13 patients died from unrelated causes within a mean of 24.9 days from the onset of bacteremia.

Five patients had two episodes of bacteremia during their hospital stay; of those three died, two of which were from causes related to the bacteremia.

Active treatment description is shown in **Table 5**. Active antibiotics were administered in 32 episodes in thirty patients within 24 h of positive culture results. Of those patients 24 died. Eight patients died prior to initiation of appropriate treatment.

Risk Factors for Mortality

Characteristics of patients stratified by mortality are shown in **Table 6**.

No significant difference in demographic characteristics, XDR profile and sources of bacteremia between survivors and non-survivors was found. Patients who did not survive had a longer pre-acquisition median hospital LOS than patients who survived (22.41 vs. 12.3 days, $p = 0.002$). Deceased patients were also more likely to have diabetes mellitus, prior exposure to high dose steroids, received colistin and/or tigecycline PTA, have septic shock at time of bacteremia, and to have stayed longer in a CC unit, with a higher rate of mechanical ventilator use.

In the multivariate analysis, the risk factors independently associated with mortality in patients with AB bacteremia were high dose steroids and septic shock (**Table 7**).

The appropriateness of antimicrobial therapy was not associated with a better outcome since 80% of the patients who had received appropriate antimicrobial therapy within 24 h of positive culture results died compared to 46.8% of those who did not ($p = 0.003$).

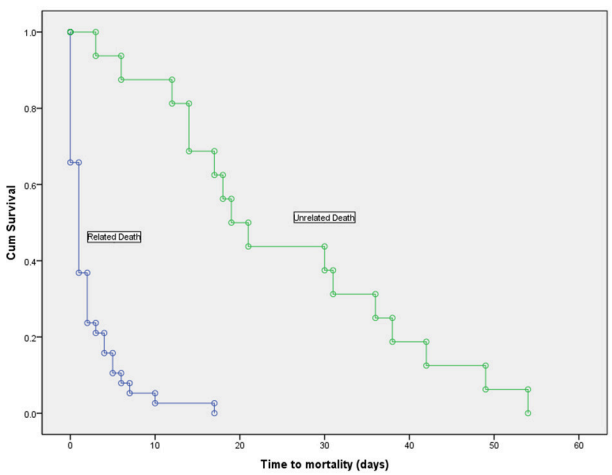


FIGURE 3 | Kaplan-Meier survival curve in deceased patients with *Acinetobacter baumannii* bacteremia, showing a significantly shorter time to mortality in patients who died due to the bacteremia vs. those who died from unrelated causes (log-rank test, $p < 0.001$).

TABLE 5 | Active antibiotics used in the treatment of *Acinetobacter baumannii* bacteremia.

Treatment	Number ($n = 32$)
Colistin	18
Tigecycline	9
Colistin and Tigecycline	5

DISCUSSION

In our study, we aimed to identify the clinical characteristics and prognostic factors in patients with AB bacteremia.

In concordance with previous reports (Esterly et al., 2011; Chopra et al., 2013, 2014; Nutman et al., 2014; Fu et al., 2015; Gulen et al., 2015; Gu et al., 2016; Guo et al., 2016; Liu et al., 2016), we found that patients with compromised baseline mobility who are barely capable of carrying any life activities as by their ECOG, with longer hospital stay especially in the critical care units, with

TABLE 6 | Demographic and clinical characteristics of patients with *Acinetobacter baumannii* bacteremia stratified by all cause in hospital mortality ($N = 90$).

Characteristics	Survivors	Non-survivors	*p-values*
Demographics			
Age, mean, year (SD)	67.33 ±13.99	71.85 ±16.77	
Male sex (%)	22	33	NS
LOS prior to event, median, days (SD)	12.3 ±11.65	22.41 ±18.05	0.002
Comorbidities (%)			
Hypertension	22	39	NS
Malignancies			
Hematologic	6	13	NS
Solid	10	11	NS
Diabetes mellitus	5	21	0.044
Coronary artery disease	11	14	NS
Congestive heart failure	4	6	NS
Renal disease	2	13	NS
Dyslipidemia	4	7	NS
Autoimmune diseases	2	4	NS
Lung disease	7	11	NS
Cerebral vascular accident	2	4	NS
Neutropenia	3	10	NS
High dose steroids	9	44	<0.001
Invasive procedures prior to event (%)			
Foley	21	47	NS
Central lines	19	49	NS
Nasogastric tube	11	29	NS
Mechanical ventilation	8	32	0.005
Drains	7	12	NS
Tracheostomy	1	6	NS
Total parenteral nutrition	5	11	NS
CC unit stay	16	44	0.03
XDR profile	28	54	NS
Infection source			
Primary	22	35	NS
Secondary	10	23	
Prior exposure to colistin and/or tigecycline	4	21	0.019
Septic shock at time of acquisition	2	39	<0.001

SD, standard deviation; LOS, length of stay; CC, critical care.

TABLE 7 | Logistic regression analysis of predictors on all-cause in-hospital mortality among patients with *Acinetobacter baumannii* bacteremia.

Predictors	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P*	OR (95%CI)	*p*
Septic shock	44.2 (9.42–207.3)	< 0.001	90.91(11.45–719.12)	< 0.001
High dose steroids	6.31 (2.48–16.02)	< 0.001	7.74 (1.634–36.6)	0.01
Prior exposure to colistin &/or tigecycline	3.94 (1.19–12.99)	0.019		
CC unit stay	3.13 (1.25–7.83)	0.03		
Mechanical ventilation	3.89 (1.54–9.99)	0.004		
Diabetes mellitus	2.94 (1.04–8.29)	0.041		

OR, odds ratio; CI, confidence interval; CC, critical care.

≥1 mg/kg/day of steroids and antimicrobial exposure are at risk for AB bacteremia with poor outcome.

The crude mortality rate in our series was 63.5%, comparable to the range of 30–76% reported in literature (Gulen et al., 2015; Leão et al., 2016). However, it has been difficult to distinguish between attributable mortality and that attributed to the underlying illnesses and comorbid conditions of patients. In a previous case control study, Jang et al. reported that the underlying illnesses seemed to play a more pivotal role than the infection itself as a cause of death (Jang et al., 2009). Attributable mortality in our study was 70.3%, showing that there is significant impact of the bacteremia on the outcome.

In our study, all but two patients with septic shock on acquisition of the bacteremia died, within a mean of 2.16 days. It is well-known that the presence of severe sepsis or septic shock in bacterial infections is associated with worse outcomes. However, there has been limited data describing the outcomes of sepsis in AB infections. Lahmer et al. reported six cases of severe sepsis caused by AB, with a 100% mortality rate (Lahmer et al., 2014). Similarly, Leão et al. associated AB infections with lower survival rates in septic ICU patients, when compared to other pathogens (Leão et al., 2016). Contrary to that, a recent cohort of septic ICU patients with AB bacteremia had a mortality rate of 49.6% (Shorr et al., 2014).

On the other end, we observed that a subset of patients had recovered from the bacteremia, despite little or no intervention. This has been previously described by Seifert et al. as "benign transient bacteremia" (Seifert et al., 1995). Though all cases of this transient bacteremia in our study were line related and removal of the line may had averted the sepsis cascade, the virulence factors of the bacteria and immune status of the host may have also played a significant role.

Previous studies have highlighted on the importance of a patient's immune status on the outcome, with prolonged LOS and mortality rates, particularly when a hematological malignancy is present (Nazer et al., 2015; Gu et al., 2016). Though we did not find any significant association between malignancies and mortality, exposure to high dose steroids increased the risk of dying by 7.4-folds. Townsend et al. (2015) in a recent publication similarly associated steroids with increased mortality.

Patients with prior exposure to colistin and/or tigecycline had appeared to have a worse outcome. One potential explanation is that previous antibiotic therapy might have an effect on selecting and expressing more virulent bacteria (Gordon and Wareham, 2010). However, this was not shown to be an independent risk factor on multivariate analysis, most likely due to the small sample size.

A rapid rise of resistance was noted over the study period, with an overall XDR rate of AB isolates of 87.7%. Yet, we could not correlate between drug resistance and mortality, which could be due to the small number of patients with non XDR AB. In literature, the effect of antibiotic resistance on survival is still a subject of debate. While some authors have argued against any attributed mortality due to resistance (Gu et al., 2016), others have suggested that resistant AB may lower survival either directly by enhanced virulence, or indirectly by delaying the onset of active antimicrobial treatment (Esterly et al., 2011; Huang et al., 2012; Lee et al., 2012; Liu et al., 2015).

In our cohort, administration of appropriate treatment within 24 h of bacteremia was associated with worse outcome. Even when XDR AB was highly suspected, the initiation of active antibiotics did not affect the outcome as much as the critical septic condition of the patient, due to limited time. On the other hand, a subset of non-septic patients who grew AB in blood cultures taken as part of the workup of fever in a hospitalized patient and did not receive active treatment against XDR AB, remained in relatively good clinical conditions. These two extremes of clinical outcome made the analysis of the effect of appropriate antibiotics complicated and may be the reason for opposing results in literature. Lee et al. postulated that inappropriate antimicrobial therapy may be less detrimental in patients who are not severely ill and in the most severely ill with short life expectancies (Lee et al., 2012). Similar results were reported by Lim et al that suggested that host factors and severity of infections reflected by APACHE are the main determinants of the outcome rather than the use of active therapy (Lim et al., 2011). Contrary to that, others have suggested that inactive microbial therapy in patients with AB bacteremia is associated with mortality, and that early initiation of appropriate antimicrobial therapy would lead to a better outcome (Nutman et al., 2014; Shorr et al., 2014).

The present study has several limitations that must be acknowledged, mainly due to its retrospective nature and small sample size. However, to eliminate potential biases, we gathered all the data needed from computerized records through a standardized data collection sheet and performed multivariate

analysis to discover independent risk factors. The presence of malignancies in 44.6% of our study population might be an overrepresentation of our hospitalized patients, in the absence of a control group. Also, as a single center study, there is lack of generalizability and may not reflect on other different institutions.

In conclusion, a prolonged hospital stay is frequently associated with loss of functionality, and steroid and antibiotic exposure. These factors seem to impact the mortality of AB bacteremia, a disease with high mortality rate and limited therapeutic options. We believe that improving outcome seems to lie more in the prevention of acquisition of AB bacteremia rather than in treating the consequences.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Good Clinical Practice Guidelines" as defined by the U.S. Food and Drug Administration under the code of Federal Regulations (21CFR Parts 50 and 56; 45 CFR Part 46) and International Conference on Harmonization (ICH) Guidelines (Section E6) and applicable local laws and regulations. The Institutional Review Board at University of Balamand-Faculty of Medicine and Medical Sciences approved this study protocol and granted a waiver of informed consent due to the retrospective nature of the study; where data collection occurred after the patients left the hospital or died. Patient medical records and information were anonymized and de-identified prior to analysis.

AUTHOR CONTRIBUTIONS

EA and CA designed the study; JA gathered the data, JI, CL, and TB analyzed the data; TB wrote the main manuscript text and prepared figures. All authors reviewed the manuscript.

REFERENCES

Asim, P., Naik, N. A., Muralidhar, V., Vandana, K. E., and Varsha, A. P. (2016). Clinical and economic outcomes of Acinetobacter vis a vis non-Acinetobacter infections in an Indian teaching hospital. *Perspect. Clin. Res.* 7, 28–31. doi: 10.4103/2229-3485.173778

Chopra, T., Marchaim, D., Awali, R. A., Krishna, A., Johnson, P., Tansek, R., et al. (2013). Epidemiology of bloodstream infections caused by *Acinetobacter baumannii* and impact of drug resistance to both carbapenems and ampicillin-sulbactam on clinical outcomes. *Antimicrob. Agents Chemother.* 57, 6270–6275. doi: 10.1128/AAC.01520-13

Chopra, T., Marchaim, D., Johnson, P. C., Awali, R. A., Doshi, H., Chalana, I., et al. (2014). Risk factors and outcomes for patients with bloodstream infection due to *Acinetobacter baumannii*-calcoaceticus complex. *Antimicrob. Agents Chemother.* 58, 4630–4635. doi: 10.1128/AAC.02441-14

Esterly, J. S., Griffith, M., Qi, C., Malczynski, M., Postelnick, M. J., and Scheetz, M. H. (2011). Impact of carbapenem resistance and receipt of active antimicrobial therapy on clinical outcomes of *Acinetobacter baumannii* bloodstream infections. *Antimicrob. Agents Chemother.* 55, 4844–4849. doi: 10.1128/AAC.01728-10

Freire, M. P., de Oliveira Garcia, D., Garcia, C. P., Campagnari Bueno, M. F., Camargo, C. H., Kono Magri, A. S., et al. (2016). Bloodstream infection caused by extensively drug-resistant *Acinetobacter baumannii* in cancer patients: high mortality associated with delayed treatment rather than with the degree of neutropenia. *Clin. Microbiol. Infect.* 22, 352–358. doi: 10.1016/j.cmi.2015.12.010

Fu, Q., Ye, H., and Liu, S. (2015). Risk factors for extensive drug-resistance and mortality in geriatric inpatients with bacteremia caused by *Acinetobacter baumannii*. *Am. J. Infect. Control.* 43, 857–860. doi: 10.1016/j.ajic.2015.03.033

Gordon, N. C., and Wareham, D. W. (2010). Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int. J. Antimicrob. Agents* 35, 219–226. doi: 10.1016/j.ijantimicag.2009.10.024

Gu, Z., Han, Y., Meng, T., Zhao, S., Zhao, X., Gao, C., et al. (2016). Risk factors and clinical outcomes for patients with *Acinetobacter baumannii* bacteremia. *Medicine (Baltimore)* 95:e2943. doi: 10.1097/MD.0000000000002943

Gulen, T. A., Guner, R., Celikbilek, N., Keske, S., and Tasyaran, M. (2015). Clinical importance and cost of bacteremia caused by nosocomial multi drug resistant *Acinetobacter baumannii*. *Int. J. Infect. Dis.* 38, 32–35. doi: 10.1016/j.ijid.2015.06.014

Guo, N., Xue, W., Tang, D., Ding, J., and Zhao, B. (2016). Risk factors and outcomes of hospitalized patients with blood infections caused by multidrug-resistant *Acinetobacter baumannii* complex in a hospital of Northern China. *Am. J. Infect. Control* 44, e37–e39. doi: 10.1016/j.ajic.2015.11.019

Huang, S. T., Chiang, M. C., Kuo, S. C., Lee, Y. T., Chiang, T. H., Yang, S. P., et al. (2012). Risk factors and clinical outcomes of patients with carbapenem-resistant *Acinetobacter baumannii* bacteremia. *J. Microbiol. Immunol. Infect.* 45, 356–362. doi: 10.1016/j.jmii.2011.12.009

Jang, T. N., Lee, S. H., Huang, C. H., Lee, C. L., and Chen, W. Y. (2009). Risk factors and impact of nosocomial *Acinetobacter baumannii* bloodstream infections in the adult intensive care unit: a case-control study. *J. Hosp. Infect.* 73, 143–150. doi: 10.1016/j.jhin.2009.06.007

Lahmer, T., Messer, M., Schnappauf, C., Schmidt, A., Schmid, R. M., and Huber, W. (2014). *Acinetobacter baumannii* sepsis is fatal in medical intensive care unit patients: six cases and review of literature. *Anaesth. Intensive Care* 42, 666–668.

Leão, A. C., Menezes, P. R., Oliveira, M. S., and Levin, A. S. (2016). Acinetobacter spp. are associated with a higher mortality in intensive care patients with bacteremia: a survival analysis. *BMC Infect. Dis.* 16:386. doi: 10.1186/s12879-016-1695-8

Lee, N. Y., Lee, H. C., Ko, N. Y., Chang, C. M., Shih, H. I., Wu, C. J., et al. (2007). Clinical and economic impact of multidrug resistance in nosocomial *Acinetobacter baumannii* bacteremia. *Infect. Control Hosp. Epidemiol.* 28, 713–719. doi: 10.1086/517954

Lee, Y. T., Kuo, S. C., Yang, S. P., Lin, Y. T., Tseng, F. C., Chen, T. L., et al. (2012). Impact of appropriate antimicrobial therapy on mortality associated with *Acinetobacter baumannii* bacteremia: relation to severity of infection. *Clin. Infect. Dis.* 55, 209–215. doi: 10.1093/cid/cis385

Lim, S. K., Lee, S. O., Choi, S. H., Choi, J. P., Kim, S. H., Jeong, J. Y., et al. (2011). The outcomes of using colistin for treating multidrug resistant Acinetobacter species bloodstream infections. *J. Korean Med. Sci.* 26, 325–331. doi: 10.3346/jkms.2011.26.3.325

Liu, C. P., Shih, S. C., Wang, N. Y., Wu, A. Y., Sun, F. J., Chow, S. F., et al. (2016). Risk factors of mortality in patients with carbapenem-resistant *Acinetobacter baumannii* bacteremia. *J. Microbiol. Immunol. Infect.* 49, 934–940. doi: 10.1016/j.jmii.2014.10.006

Liu, Q., Li, W., Du, X., Zhong, T., Tang, Y., Feng, Y., et al. (2015). Risk and prognostic factors for multidrug-resistant *Acinetobacter baumannii* complex bacteremia: a retrospective study in a tertiary hospital of West China. *PLoS ONE* 10:e0130701. doi: 10.1371/journal.pone.0130701

Munoz-Price, L. S., and Weinstein, R. A. (2008). Acinetobacter infection. *N. Engl. J. Med.* 358, 1271–1281. doi: 10.1056/NEJMra070741

Ñamendys-Silva, S. A., Correa-García, P., García-Guillén, F. J., González-Herrera, M. O., Pérez-Alonso, A., Texcocano-Becerra, J., et al. (2015). Outcomes of critically ill cancer patients with *Acinetobacter baumannii* infection. *World J. Crit. Care Med.* 4, 258–264. doi: 10.5492/wjccm.v4.i3.258

Nazer, L. H., Kharabsheh, A., Rimawi, D., Mubarak, S., and Hawari, F. (2015). Characteristics and outcomes of *Acinetobacter baumannii* infections in critically ill patients with cancer: a matched case-control study. *Microb. Drug Resist.* 21, 556–561. doi: 10.1089/mdr.2015.0032

Nutman, A., Glick, R., Temkin, E., Hoshen, M., Edgar, R., Braun, T., et al. (2014). A case-control study to identify predictors of 14-day mortality following carbapenem-resistant *Acinetobacter baumannii* bacteraemia. *Clin. Microbiol. Infect.* 20, O1028–O1034. doi: 10.1111/1469-0691.12716

Seifert, H., Strate, A., and Pulverer, G. (1995). Nosocomial bacteremia due to *Acinetobacter baumannii*. Clinical features, epidemiology, and predictors of mortality. *Medicine (Baltimore)* 74, 340–349.

Shorr, A. F., Zilberberg, M. D., Micek, S. T., and Kollef, M. H. (2014). Predictors of hospital mortality among septic ICU patients with Acinetobacter spp. bacteremia: a cohort study. *BMC Infect. Dis.* 14:572. doi: 10.1186/s12879-014-0572-6

Sunenshine, R. H., Wright, M. O., Maragakis, L. L., Harris, A. D., Song, X., Hebden, J., et al. (2007). Multidrug-resistant Acinetobacter infection mortality rate and length of hospitalization. *Emerging Infect. Dis.* 13, 97–103. doi: 10.3201/eid1301.060716

Townsend, J., Park, A. N., Gander, R., Orr, K., Arocha, D., Zhang, S., et al. (2015). Acinetobacter infections and outcomes at an academic medical center: a disease of long-term care. *Open Forum Infect. Dis.* 2:ofv023. doi: 10.1093/ofid/ofv023

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

REVIEW
published: 15 February 2017
doi: 10.3389/fcimb.2017.00039

Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence

*M. Fata Moradali †, Shirin Ghods and Bernd H. A. Rehm **

Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand

Pseudomonas aeruginosa is an opportunistic pathogen affecting immunocompromised patients. It is known as the leading cause of morbidity and mortality in cystic fibrosis (CF) patients and as one of the leading causes of nosocomial infections. Due to a range of mechanisms for adaptation, survival and resistance to multiple classes of antibiotics, infections by *P. aeruginosa* strains can be life-threatening and it is emerging worldwide as public health threat. This review highlights the diversity of mechanisms by which *P. aeruginosa* promotes its survival and persistence in various environments and particularly at different stages of pathogenesis. We will review the importance and complexity of regulatory networks and genotypic-phenotypic variations known as adaptive radiation by which *P. aeruginosa* adjusts physiological processes for adaptation and survival in response to environmental cues and stresses. Accordingly, we will review the central regulatory role of quorum sensing and signaling systems by nucleotide-based second messengers resulting in different lifestyles of *P. aeruginosa*. Furthermore, various regulatory proteins will be discussed which form a plethora of controlling systems acting at transcriptional level for timely expression of genes enabling rapid responses to external stimuli and unfavorable conditions. Antibiotic resistance is a natural trait for *P. aeruginosa* and multiple mechanisms underlying different forms of antibiotic resistance will be discussed here. The importance of each mechanism in conferring resistance to various antipseudomonal antibiotics and their prevalence in clinical strains will be described. The underlying principles for acquiring resistance leading pan-drug resistant strains will be summarized. A future outlook emphasizes the need for collaborative international multidisciplinary efforts to translate current knowledge into strategies to prevent and treat *P. aeruginosa* infections while reducing the rate of antibiotic resistance and avoiding the spreading of resistant strains.

Keywords: ***Pseudomonas aeruginosa*, virulence, biofilm, antibiotic resistance, persistence**

OPEN ACCESS

Edited by:
Ghassan M. Matar, American University of Beirut, Lebanon

Reviewed by:
Eduard Torrents, Institute for Bioengineering of Catalonia, Spain
Xingmin Sun, University of South Florida, USA

***Correspondence:**
Bernd H. A. Rehm
b.rehm@massey.ac.nz

†Present Address:
M. Fata Moradali, Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL, USA

Received: *20 December 2016*
Accepted: *02 February 2017*
Published: *15 February 2017*

Citation:
Moradali MF, Ghods S and Rehm BHA (2017) Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. Front. Cell. Infect. Microbiol. 7:39. doi: 10.3389/fcimb.2017.00039

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative and ubiquitous environmental bacterium. It is an opportunistic human pathogen capable of causing a wide array of life-threatening acute and chronic infections, particularly in patients with compromised immune defense. It has been of particular importance since it is the main cause of morbidity and mortality in cystic fibrosis (CF) patients and one of the leading nosocomial pathogens affecting hospitalized patients while being intrinsically resistant to a wide range of antibiotics.

P. aeruginosa strains possess large genomes (∼5–7 Mbp). Their metabolic capacity is extensive as exemplified by their ability to produce multiple secondary metabolites and polymers as well as their ability to use various carbon sources and electron acceptors. The repertoire of *P. aeruginosa* genes which are substantially conserved suggest the highest proportion of regulatory genes and networks observed in known bacterial genomes and is foundational to respond and adapt to diverse environments (Stover et al., 2000; Mathee et al., 2008; Frimmersdorf et al., 2010). The ubiquitous presence of *P. aeruginosa* as well as its prevalence and persistence in clinical settings including intrinsic resistance to therapeutics are attributed to its extraordinary capability of survival by recruiting an arsenal of responsive mechanisms.

In the present review, we have attempted to summarize the diversity of these mechanisms causing the versatility of *P. aeruginosa* to adapt and thrive in unfavorable conditions particularly during pathogenesis. To this end, we will describe the clinical importance of *P. aeruginosa* followed by the well-characterized and most recent findings about key strategic adaptation mechanisms including quorum sensing (QS), motility-sessility switch, biofilm formation, antibiotic resistance mechanisms, adaptive radiation for persistence, stringent response and persisters, and the CRISPR-Cas systems. Recent findings on adaptive mechanisms will be set into context of the overall physiology of *P. aeruginosa* by also depicting on future research needs.

CLINICAL IMPORTANCE

The CF patients suffer from a multisystem disease due to inheritable genetic defects in the CF transmembrane conductance regulator (*CFTR*) gene. However, the recurrence of bacterial infections in the abnormal mucus layers is the main cause of morbidity and mortality of CF patients (Khan et al., 1995; Rosenfeld et al., 2001). The CFTR regulator is responsible for regulating the transport of electrolytes and chloride across epithelial cell membranes to maintain normal mucus properties and homeostasis. Therefore, the loss of function of the CFTR protein results in abnormally thick, dehydrated and sticky mucus layers in the lung (Flume and Van Devanter, 2012). Hence, the CF patients are largely susceptible to respiratory infections by *P. aeruginosa* from infancy. When they are under a year old, almost 30% of CF infants can acquire initial *P. aeruginosa* strains from the environment leading to acute infections. This rate increases to about 50% by the age of 3 years while mucoid phenotypes causing chronic infections have been reported emerging at the age of 3 to16 years (median of 13 years) (Rehm et al., 1994; da Silva et al., 2013; Jones et al., 2016). *P. aeruginosa* will adapt to CF airways and persist as overwhelming, predominant and ineradicable infections to the end of patients' life in almost 70% of adults (Döring et al., 2000).

Furthermore, *P. aeruginosa* is also largely associated with hospital acquired infections including ventilator-associated pneumonia, central line-associated bloodstream infection, urinary catheter-related infection, and surgical/transplantation infections (Cardo et al., 2004; Nathwani et al., 2014; Trubiano and Padiglione, 2015). The International Nosocomial Infection Control Consortium reported that *P. aeruginosa* nosocomial infections have become a worldwide healthcare issue (Rosenthal et al., 2016). A cohort study reported that *P. aeruginosa* had the highest burden of healthcare-acquired infections in European intensive care units (Lambert et al., 2011). In the United States healthcare-associated *P. aeruginosa* infections were estimated to contribute to 51,000 cases each year (Eurosurveillance Editorial Team, 2013). *P. aeruginosa* is prevalent in healthcare settings because it is a common companion of patients under medical care and also it can survive on abiotic and biotic surfaces such as medical equipment resisting disinfection methods while being transmissible from patient-to-patient (Russotto et al., 2015).

P. aeruginosa infections are becoming more difficult to treat because this bacterium is naturally resistant to many antibiotics and the number of multidrug- and pan-drug-resistant strains is increasing worldwide. Strains have been reported which are resistant to almost all class of commonly used antibiotics including aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems (Hancock and Speert, 2000; Poole, 2011; Eurosurveillance Editorial Team, 2013). In the United States about 13% of *P. aeruginosa* infections are caused by multidrug resistant strains (Eurosurveillance Editorial Team, 2013).

P. aeruginosa utilizes sophisticated genotypic events to support various phenotypes and molecular mechanisms required for survival during pathogenesis and antibiotic treatment.

Therefore, at initial stages of CF lung colonization, a large number of virulence and intrinsic antibiotic resistance mechanisms mediate survival. After infection, bacteria are exposed to inflammatory responses including oxidative stress followed by treatment with antibiotics (Furukawa et al., 2006; Turner et al., 2014). These environmental stress factors induce the expression of different sets of genes enabling *P. aeruginosa* to adapt and switch to persisting and resistant phenotypes, while becoming less virulent, such as upon formation of mucoid biofilms (MacDougall et al., 2005; Poole, 2012; Gellatly and Hancock, 2013). Due to the existence of an arsenal of molecular mechanisms conferring resistance to multiple classes of antibiotics, therapeutic options are increasingly limited for treatment of infections, while the number of infection incidences and multi-drug resistance strains are increasing.

CENTRAL REGULATORY ROLE OF QUORUM SENSING (QS) FOR VIRULENCE AND ADAPTATION

Communication between individual cells using specific chemical signals is a well-known capability of bacteria and is called quorum sensing. Indeed, QS controls social behavior of bacteria by multiple interconnected signaling pathways (LaSarre and Federle, 2013). It allows bacterial communities to regulate a variety of biological processes important for bacterial adaptation and survival. Basically, this phenomenon relies on regulating the expression of specific sets of genes in response to a critical threshold of signaling molecules known as autoinducers

(AIs). QS will mediate population density dependent collective responses and is therefore beneficial for community survival. A study showed that cells' responses to QS signals and the corresponding gene expression profile is heterogeneous within a given community leading to increasing fitness and chance of survival (Grote et al., 2015).

During pathogenesis *P. aeruginosa* QS plays a critical role for survival and colonization by coordinating phenotypic alterations at early stages of infection i.e., after attachment (González and Keshavan, 2006). The progress of acute to chronic infection is critically influenced by QS-dependent gene expression. More than 10% of *P. aeruginosa* genes are regulated by QS. These genes are mainly involved in virulence factor production, motility, motility-sessility switch and biofilm development, antibiotic resistance mechanisms and the adjustment of metabolic pathways for stress responses (Venturi, 2006; Williams and Camara, 2009; Barr et al., 2015). The role of QS in each physiological adaptation will be discussed below.

Molecular Mechanisms Underlying QS

As shown in **Figure 1**, four main pathways of QS dependent signaling exist in *P. aeruginosa*. These constitute a hierarchal network mediating integration of multiple signals via cross-talk between the QS signaling pathways. The most recently discovered IQS signaling pathway is less understood and its integration and impact on gene expression still needs to be unraveled. It was previously proposed that the IQS molecule (an aeruginaldehyde) is the product of enzymatic activity of proteins encoded by *ambBCDE* genes (Lee et al., 2013), while new findings showed the IQS molecule is a byproduct of the pyochelin biosynthesis pathway and AmbBCDE proteins are responsible for the biosynthesis of the toxin L-2-amino-4-methoxy-trans-3-butenoic acid (AMB) (Ye et al., 2014; Rojas Murcia et al., 2015; Sun et al., 2016).

In regard to the other three QS pathways, each system consists of at least two major functional elements; the first category of proteins (i.e., LasR, RhlR, or PqsR, respectively) is activated upon sensing specific autoinducers (AIs) and acts as transcriptional activator for genes encoding the second tier proteins, the cognate AI synthases (i.e., LasI, RhlI, and PqsABCDH, respectively). These activation steps constitute a fine-tuned circuit by which the synthesized AIs are exported outside the cells followed by being imported again (**Figure 1**). Transportation of these signals is not well understood, but it is proposed as being mediated by free diffusion, membrane transporters such as specific efflux systems or membrane vesicles (Mashburn and Whiteley, 2005; Martinez et al., 2009; Alcalde-Rico et al., 2016). Beyond this regulatory circuit the activated AI-sensing proteins act as transcriptional factors for activating the expression of other set of genes such as virulence genes in response to environmental stimuli (**Figure 1**). Transcriptional activation occurs via binding to conserved *las/rhl* boxes acting as operators residing upstream of these genes (González-Valdez et al., 2014; Lee and Zhang, 2015; Banerjee and Ray, 2016; Papenfort and Bassler, 2016). In the hierarchy of this network, LasR resides at the top of the cascade and along with RhlR mediates QS signaling at early stages of exponential growth phase while the PQS signaling pathway is active at late exponential growth phase (Choi et al., 2011). As abovementioned, cumulative and cell density-dependent production of AIs is required for reaching a specific threshold triggering collective responses by individual cells.

QS-Controlled Virulence Factors and Stress Responses

Production of virulence factors is a survival strategy for pathogens to evade the host immune defense resulting in progression of pathogenesis particularly at early stage of colonization and acute infection. A large number of virulence factors including cell-associated or secreted compounds, both low and high molecular weight compounds have been reported as important in colonization and establishment of infections by *P. aeruginosa*. Although they play a critical role in promoting bacterial growth and survival, they can cause devastating injuries to the host tissues and impair the immune responses. QS deficient mutants cause considerably less tissue damage and pathological changes during infections due to a significant decrease in the virulence and cytotoxicity (Nelson et al., 2009; Feng et al., 2016).

Production of many virulence factors is metabolically costly and requires community involvement. Hence, they are mainly under the regulatory control of the QS systems (**Figure 1**, **Table 1**; Whiteley et al., 1999; Diggle et al., 2002; Wagner et al., 2003; García-Contreras, 2016).

Analysis of bronchial secretions of CF patients during different stages of pulmonary exacerbations showed that QS upregulates the expression of genes involved in the production of some destructive virulence factors such as proteases (elastase, alkaline protease), phenazines (pyocyanin), toxins (exotoxin A), rhamnolipids and hydrogen cyanide (Jaffar-Bandjee et al., 1995; Lee and Zhang, 2015). Production of these toxic compounds is destructive to the host cells/tissues by impairing permeability barrier and by inhibiting protein production promoting cell death.

Recent findings suggested a correlation between systemic concentrations of some QS signaling molecules with the clinical status of pulmonary exacerbation and at least some QS signaling molecules were elevated at the start of either pulmonary exacerbation or antibiotic treatment when assessing different biofluids (Barr et al., 2015). In conclusion, virulence factors assist bacteria in colonization and survival aligned with worsened clinical course of infections. Thus, QS can determine the degree of pathological damages and clinical stages of infections in response to environmental factors.

Pathogenesis encompasses various stresses such as host immune factors, bacterial interspecies competition, phosphate/iron depletion and starvation. QS systems and production of some virulence factors mediate appropriate responses to these stresses to promote survival and adaptation (García-Contreras et al., 2015). Here, we provide some examples of stress responses mediated by the QS systems.

Interferon-γ (IFN-γ) is a crucial cytokine of the human immune system during infection and it coordinates a wide array of immunological responses such as up-regulation of pathogen recognition and the activation of bactericidal effector functions

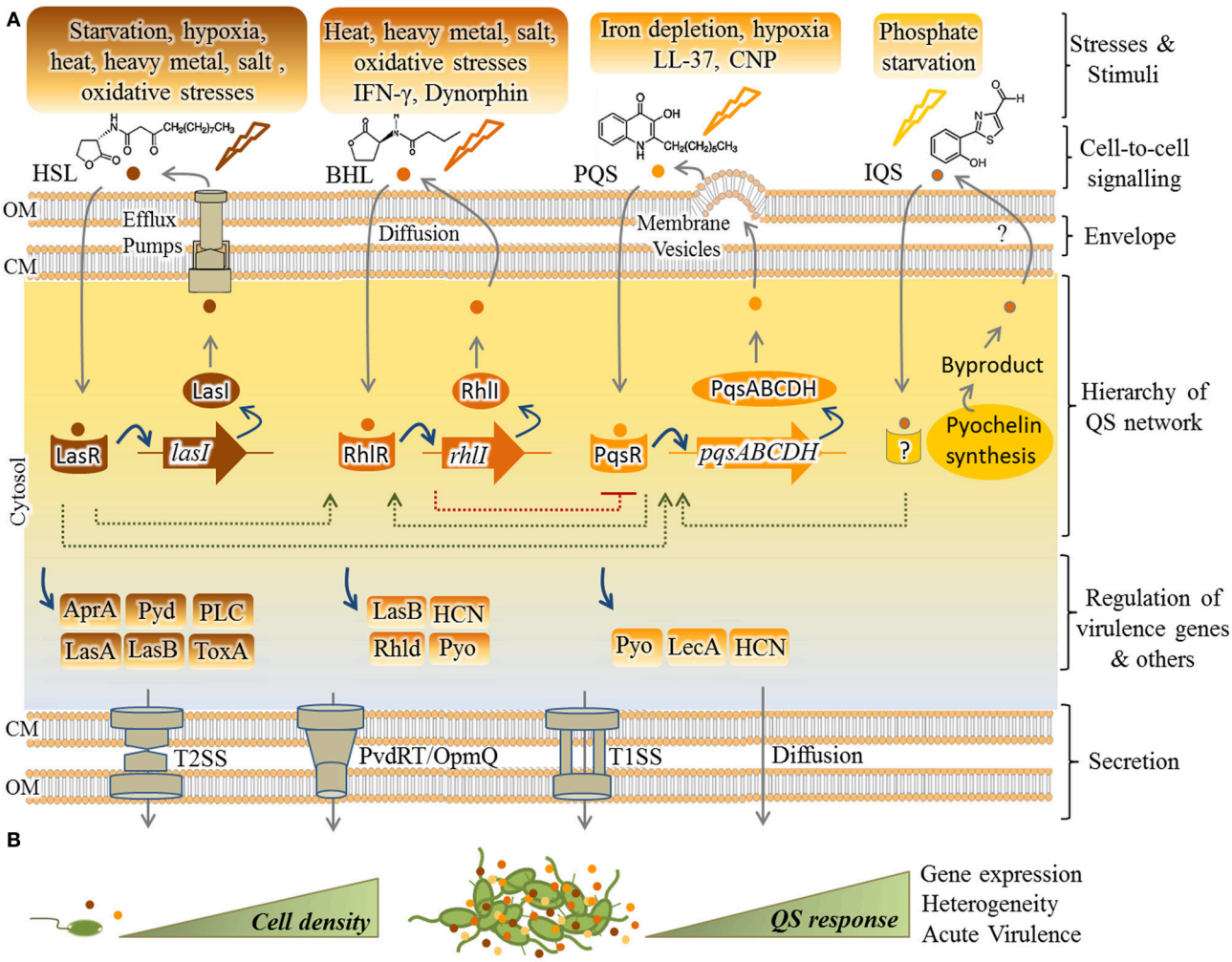


FIGURE 1 | Hierarchical QS network in *P. aeruginosa* and regulation of virulence factors. (A) So far, four pathways including Las, Rhl, Pqs, and IQS have been understood as mediating QS responses in *P. aeruginosa* while LasR resides at the top of the cascade. In response to specific stimuli/stress, each pathway synthesizes cognate autoinducers (AIs) [HSL(3-oxo-C12-homoserine lactone)], BHL (*N*-butyrylhomoserine lactone or C4-HSL), PQS (2-heptyl-3-hydroxy-4-quinolone) and IQS [2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (aeruginaldehyde)]. Export and import of HSL, BHL, and PQS is mediated by the efflux pumps MexAB-OprM/MexEF-OprN, free diffusion and membrane vesicles, respectively. Question mark indicates unknown pathway of IQS transportation. As fine-tuned individual circuits, but interconnected (dashed lines), transcriptional factors (i.e., LasR, RhlR, and PqsR) are activated by AIs for upregulating expression of cognate AI synthases (respectively, LasI, RhlI, PqsABCDH) as well as others such as virulence factor genes. The IQS pathway remains unraveled and the IQS receptor is still unknown. Various secretion systems mainly type 1 and 2 secretion systems (T1SS/T2SS) and also PvdRT-OpmQ efflux pump mediate the secretion of virulence factors. **(B)** QS initiates upon cumulative production of AIs (small colorful circles) by increasing cell density and results in collective responses. AprA, alkaline protease; Pyd, pyoverdine; PLC, phospholipase C; Tox, toxin A; LasA, LasA elastase; LasB, LasB elastase; HCN, hydrogen cyanide; Pyo, pyocyanin; Rhld, rhamnolipids; Lec A, lectin A; CM, cytoplasmic membrane; OM, outer membrane.

(Schroder et al., 2004). IFN-γ produced by T-cells was shown to bind directly to *P. aeruginosa* OprF, an outer membrane protein. Upon formation of IFN-γ-OprF complexes the *rhl* QS system was activated and resulted in up-regulation of the expression of *lecA* (or PA-I lectin) and synthesis of pyocyanin. The *lecA* gene encodes the virulence determinant, galactophilic lectin (or LecA) (Wu et al., 2005) which is cytotoxic and acts as adhesion factor mediating initiation of host recognition by *P. aeruginosa* (Chemani et al., 2009). It induces an increased permeability of the intestinal and respiratory epithelial cells enabling cytotoxic exoproducts such as exotoxin A (Laughlin et al., 2000) to enter host cells (Bajolet-Laudinat et al., 1994). In addition it also contributes to biofilm development (Diggle et al., 2006). Furthermore, the QS system has been reported to mediate a response to the host antimicrobial factor LL-37 by increasing the production of pyocyanin, hydrogen cyanide, elastase and rhamnolipids (Strempel et al., 2013). The QS-dependent production of rhamnolipids has a crucial role in neutralizing the attack of neutrophils due to their necrotic property (Jensen et al., 2007; Van Gennip et al., 2009).

Recent findings indicated that the LasR and RhlR QS systems, but not the PQS system, play major roles in adaptation and

TABLE 1 | Key QS-dependent virulence factors produced by *P. aeruginosa*.

Virulence factor	Class/Chemistry	Synthesis	Secretion	Property	Role in pathogenesis	References
Pyocyanin	Secondary metabolite/ tricyclic phenazine	*phzA1-G1* & *phzA2-G2* operons	T2SS	Redox-active, zwitterion	Cytotoxic/damaging host cells, tissues and immune system cells/inducing apoptosis/ causing oxidative stress by mediating O_2^- and H_2O_2 production	Britigan et al., 1992; Li et al., 2011; van 't Wout et al., 2015; Hall et al., 2016
Pyoverdine	Pyoverdines/ dihydroquinoline-type chromophore linked to a peptide	Large multimodular enzymes/ non-ribosomal peptide synthetases (NRPSs)	PvdRT-opmQ Efflux pump & MexAB-OprM efflux pumps	High affinity to Fe(III)/ iron acquisition/ fluorescent	Carrier of iron and other metals /crucial for infection and biofilm development	Visca et al., 2007; Hannauer et al., 2012; Schalk and Guillon, 2013
LasA Elastase	β-lytic zinc metallo-endopeptidases (staphylolytic)/ serine protease	*lasA*	The Sec pathway & T2SS	Protease and elastolytic activity/ cleaving a wide range of glycine-containing proteins	Staphylolytic activity/ enhancing the activity of LasB and host elastolytic proteases/ crucial for tissue invasion and infection	Toder et al., 1994; Kessler et al., 1997; Hoge et al., 2010
LasB Elastase	M4 thermolysin peptidase family/ zinc metalloprotease	*lasB*	The Sec pathway & T2SS	Protease and elastolytic activity	Degrading host proteins (e.g., elastin, collagen and fibrin)/ damaging host tissues/ inactivating key components of the immune systems/ Corneal amage/crucial for tissue invasion and infection	Toder et al., 1994; Hoge et al., 2010
Alkaline Protease (aeruginolysin)	M10 peptidase family/zinc-metalloendopeptid-ase	*aprA*	T1SS	Wide protease activity	Degrading tissue proteins such as laminin/destroying basal lamina/ causing hemorrhagic tissue necrosis/ inactivating key components of the immune systems/ crucial for tissue invasion	Guzzo et al., 1991; Hoge et al., 2010; Laarman et al., 2012
Lectin A	Tetrameric protein	*lecA* (or *pa1L*)	Intracellular; only a small fraction present on the cell surface	Galactophilic/ adhesive	Cytotoxic/impairing respiratory of epithelial cells/ inducing a permeability defect in the intestinal epithelium boosting ExoA penetration/ important for cell attachment, cell-cell interaction and biofilm development	Glick and Garber, 1983; Diggle et al., 2006; Chemani et al., 2009
PlcB	Phospholipases C	*plcB*	The Sec pathway & T2SS	Hydrolysing phosphatidylch-oline & phosphatidyleth-anolamine	Cytolytic activity/ important for cell membrane destruction and tissue invasion	Schmiel and Miller, 1999; Barker et al., 2004
Rhamnolipids	Rhamnose-containing glycolipidic compounds	*rhlAB* operon & *rhlC*		Biosurfactant/ detergent-like structure/ hemolytic activity	Cytotoxic/Causing tissue invasion and damage/ eliminating Polymorphonuclear neutrophilic leukocytes/ inhibiting the mucociliary transport and ciliary function of human respiratory epithelium/ important for maintenance of biofilm architecture and bacterial motility	Van Delden and Iglewski, 1998; Davey et al., 2003; Zhu and Rock, 2008; Alhede et al., 2009; Wittgens et al., 2011
Exotoxin A (ToxA)	PE belongs to the two-component AB toxin family/ NAD^+-diphthamide-ADP-ribosyltransferase	*toxA*	T2SS	Modifying the elongation factor-2 in eukaryotic cells	A systemic and one of the most toxic virulence factor/cytotoxic by entering host cells/ inhibiting host protein synthesis/ causing cell death, tissue damage, bacterial invasion and immunosuppression/ crucial for keratitis	Pillar and Hobden, 2002; Daddaoua et al., 2012; Michalska and Wolf, 2015
Hydrogen cyanide (HCN)	Secondary metabolite	*hcnABC* operon	Diffusible	Highly toxic/ potent inhibitor of cytochrome c oxidase and other metalloenzymes	Cytotoxic/Suppressing aerobic respiration by rapid diffusion through tissues	Castric, 1975; Blumer and Haas, 2000; Lenney and Gilchrist, 2011; García-García et al., 2016

response to environmental stresses such as oxidative, heat, heavy metal and salt stresses (García-Contreras et al., 2015).

The stress response of *P. aeruginosa* to the depletion of phosphate and iron was found to be linked (Slater et al., 2003). Different studies showed that acquisition of phosphate and iron are important for survival and pathogenesis of *P. aeruginosa* and the expression of cognate genes mediating acquisition of these elements are upregulated upon interaction with human respiratory epithelial cells (Frisk et al., 2004; Chugani and Greenberg, 2007). Various studies unraveled that phosphate- and iron-deficient conditions can trigger the activation of the QS system especially via the IQS- or PQS-dependent pathway leading to boosted activation of central QS and the production of virulence factors such as rhamnolipids, phenazines, cyanide, exotoxin A, LasA protease, elastase, and antimicrobials (Kim et al., 2003; Long et al., 2008; Zaborin et al., 2009; Bains et al., 2012; Lee et al., 2013; Nguyen et al., 2015). Production of such virulence factors can increase cytotoxic impact of bacteria on host tissue and promote pathogen survival.

The recently discovered IQS system which controls the expression of a large set of virulence factors was shown to be directly activated by phosphate limitation in *P. aeruginosa* (Lee et al., 2013; Lee and Zhang, 2015).

P. aeruginosa QS signaling molecules such as 2-heptyl-4-hydroxyquinoline (HHQ) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) can serve as antimicrobial agents against *Staphylococcus aureus* which is commonly present during early stages of pulmonary infections in CF patients. It is proposed that this antibacterial activity supports the dominance of *P. aeruginosa* during the course of infection. Interestingly, this inter-species competition is linked to the availability of iron as the depletion of iron potentiates the antistaphylococcal activity of these metabolites (Nguyen et al., 2016). Also, LasA is a staphylolytic protease produced by *P. aeruginosa* and it is under the regulation of the *las* QS system (Toder et al., 1991). Furthermore, when *P. aeruginosa* was grown together with the yeast *Candida albicans* in a mixed biofilm, the QS system upregulated the production of virulence factors such as pyoverdine, rhamnolipids and pyocyanin (Trejo-Hernández et al., 2014).

Oxygen depletion known as hypoxia is another stress factor for *P. aeruginosa* during pathogenesis. Hypoxia condition is influenced by various factors such as reduced ventilation through viscose layers, chronic inflammation, microbial population and biofilm formation (Hassett, 1996; Worlitzsch et al., 2002; Yoon et al., 2002; Alvarez-Ortega and Harwood, 2007; Hassett et al., 2009). However, *P. aeruginosa* can survive and grow under hypoxia to high cell densities. Under hypoxia stress *P. aeruginosa* retains the capability of microaerobic respiration, although occurrence of nitrate respiration was thought to be possible (Alvarez-Ortega and Harwood, 2007). The QS regulon expression occurs at low oxygen conditions. Hammond et al. (2015) unraveled that the 4-hydroxy-2-alkylquinolines (HAQ)-dependent QS pathway is active during hypoxia via the ANR protein as the master transcriptional regulator of anaerobic respiration while it is in the absence of LasR signaling (Hammond et al., 2015). Under low oxygen tension, the ANR protein positively regulated the production of the QS signaling molecule 4-hydroxy-2-alkylquinolines and in turn the regulation of virulence-related genes could continue via PQS system (Hammond et al., 2015). Furthermore, under hypoxia stress the ANR protein and the QS systems cooperatively regulate hydrogen cyanide biosynthesis (Castric, 1983, 1994; Pessi and Haas, 2000). This study provided further evidence that low oxygen-dependent QS inversely correlates with denitrification i.e., suppresses nitrate respiration (Hammond et al., 2015).

Overall, these examples have provided further insight into the versatility of *P. aeruginosa* to adapt to various environmental conditions by processing signals via integrated and cross-talking QS pathways resulting in enhanced survival i.e., in a medical context establishment of acute and chronic infections.

PERSISTENCE AND BIOFILM FORMATION

During acute infection the relationship between pathogen and host is reciprocally devastating as a variety of cytotoxic molecules produced by bacteria impair the host cellular processes while bacteria on the other hand encounter immune system responses such as production of antimicrobial compounds and reactive oxygen species, as well as enhanced phagocytosis. In this context, motile *P. aeruginosa* display a more virulent phenotype. Various modes of *P. aeruginosa* motility such as swimming and swarming involving flagella and twitching using type 4 pili are associated with virulent traits (Winstanley et al., 2016). A motile cell is readily detectable by the host immune system via flagellar and/or other motility components mediating recognition and induction of signaling pathways which trigger inflammatory responses and phagocytosis by murine or human macrophages (Amiel et al., 2010).

Switching to sessile lifestyle along with lower virulency is a survival advantage by which many pathogenic bacteria such as *P. aeruginosa* evade stresses and adverse conditions. They lose motility and attach to surfaces and form cellular aggregations or microcolonies which are embedded in extracellular polymeric substances (EPS) to protect bacteria from the surrounding environment. These structures are so called biofilms conferring an extreme capacity for persistence against phagocytosis, oxidative stresses, nutrient/oxygen restriction, metabolic waste accumulation, interspecies competitions, and conventional antimicrobial agents (Leid, 2009; Olsen, 2015).

Formation of mucoid biofilm by *P. aeruginosa* is the hallmark of chronic infections and indicative of disease progression and long-term persistence. As a consequence, *P. aeruginosa* dominates the microbial community of CF lungs in patients older than 24 years (McDaniel et al., 2015).

Other *P. aeruginosa* biofilm associated infections include chronic wound infection, chronic otitis media, chronic rhinosinusitis, catheter-associated urinary tract infection, and contact lens-related keratitis (Römling and Balsalobre, 2012).

Composition of the *P. aeruginosa* Biofilm

Formed on abiotic and biotic surfaces, the matrix of most biofilms embedding bacterial cells may account for over 90% of

dry weight of whole biofilm mass. In fact, this matrix creates a niche rendering bacteria for intense cell-cell interaction and communication as well as a reservoir of metabolic substances, nutrients and energy for promoting growth while shielding cells from unfavorable conditions (Flemming and Wingender, 2010). The matrix is mainly formed by extracellular polymeric substances (EPS) which are mainly polysaccharides, proteins, extracellular DNA (eDNA) and lipids (Strempel et al., 2013). Major polymers and relevant characteristics are listed in **Table 2**.

The exopolysaccharides Psl, Pel, and alginate are major constituents of the *P. aeruginosa* biofilm matrix involved in surface adhesion and together with eDNA determine the biofilm architecture. These EPS play an important role in resistance to immune responses and antibiotic treatments (Ghafoor et al., 2011; Gellatly and Hancock, 2013; Strempel et al., 2013). The differential role of each EPS has been analyzed at each stage of biofilm development. The various exopolysaccharides and eDNA were shown to interactively contribute to the biofilm architecture (Ghafoor et al., 2011). The presence of various EPS exhibiting different physiochemical properties confers a survival strategy for increasing the flexibility and stability of biofilms under various conditions (Jennings et al., 2015).

The Psl polysaccharide is a key element at early stage of biofilm formation when cells explore surfaces for adhesion (Overhage et al., 2005). It is anchored around cells in a helical arrangement initiating biofilm formation by enhancing cell migration, cell-cell interaction and cell-surface adhesion whereas in mature biofilms it is located to the periphery of mushroom shaped macrocolonies (Ma et al., 2009; Zhao et al., 2013). Psl can exist as a fiber-like matrix requiring type 4 pili-mediated migration of cells (Wang S. et al., 2013). It protects cells against phagocytosis and oxidative stress during infection (Mishra et al., 2012). Recent studies suggested that Psl can provide an instant protective role against anti-biofilm agents and a broad spectrum of antibiotics particularly at early stage of biofilm development (Zegans et al., 2012; Billings et al., 2013). Therefore, Psl provides a survival advantage during pathogenesis.

Similar to Psl, Pel is important for initiating and maintaining cell–cell interaction in biofilms (Colvin et al., 2011). Pel and/or Psl are the primary matrix structural polysaccharides in non-mucoid *P. aeruginosa* strains as a predominant environmental phenotype. However, contribution of Psl and Pel to the structure of mature biofilms is strain-dependent while both unique and functionally redundant roles have been reported for these exopolysaccharides (Colvin et al., 2012). Recent studies elucidating the chemical structure and biological function of Pel demonstrated that it is a major structural component of the biofilm stalk where it cross-links eDNA and structurally compensates for the absence of Psl in the periphery of mature biofilm (Jennings et al., 2015). Furthermore, Pel was shown to protect bacteria against certain aminoglycoside antibiotics (Colvin et al., 2011).

Overproduction of the exopolysaccharide, alginate, is characteristic for mucoid phenotype of most clinical isolates from CF patients. During adaptation to the CF lung environment, alginate is overproduced and predominantly constitutes the matrix of mature biofilms conferring a slimy or mucoid phenotype. Indeed, it is greatly important in biofilm maturation, structural stability and protection as well as persistence by shielding *P. aeruginosa* cells against opsonophagocytosis, free radicals released from immune cells, and antibiotics used for treatment (Hay et al., 2009a; Hay I. D. et al., 2013; Strempel et al., 2013). Some *in vitro* biofilm studies showed that the composition of the alginates can greatly influence biofilm characteristics such as viscoelastic property, bio-volume, cell density and architecture as well as cell-to-cell interaction, cell aggregation and surface attachment (Tielen et al., 2005; Moradali et al., 2015).

As abovementioned, eDNA is another important structural component for biofilm development and along with the Pel polysaccharide it can be detected within the stalks of mushroom-shaped macrocolonies. However, eDNA has multifaceted roles in biofilm formation such as contribution to forming cation gradients in the matrix via the chelating interaction of highly anionic DNA with cations such as Mg^{2+}, Ca^{2+}, Mn^{2+}, and Zn^{2+}, as a nutrient source during starvation, facilitating twitching motility and coordinating cell movements and conferring antibiotic resistance (Allesen-Holm et al., 2006; Mulcahy et al., 2008; Gloag et al., 2013).

Among the proteinaceous biofilm constituents, both flagella and the type 4 pili are important during maturation of the biofilm, however, these cell appendages are not commonly considered as classical matrix components of biofilms. Type 4 pili are important for adhesion and promote initial attachment of cells to surfaces at early stage of biofilm formation. Together with eDNA, flagella and the type 4 pili mediate migration required for the formation of the stalk and the cap in the mushroom-shaped microcolonies of the mature biofilm (**Table 2**; Barken et al., 2008; Mann and Wozniak, 2012).

Central Regulatory Network Governing the Motility-Sessility Switch

Transition from motility to sessility requires dynamic regulatory networks at transcriptional, post-transcriptional and post-translational levels resulting in coordinated timely expression of hundreds of genes. These events mainly arrest flagella based motility and the production of virulence factors such as exotoxins and proteases while positively regulating surface attachment, EPS production and biofilm maturation (**Figure 2**).

The small molecule cyclic-3′5′-diguanylic acid (cyclic di-GMP) is a key signal in post-transcriptional regulation of biofilm formation. It is an almost ubiquitous second messenger present in a wide range of bacteria that principally controls motility-sessility switch. The major determinant for this substantial phenotypic change is the cellular level of cyclic di-GMP, so that its elevation triggers biofilm formation while inhibiting motility. The cyclic di-GMP signaling system is very complex and two groups of proteins have been identified as main actors. The first group comprises cyclic di-GMP metabolizing enzymes including diguanylate cyclases (DGC) (containing GGDEF motif) and phosphodiesterases (containing EAL or HD-GYP motif) that respectively synthesize and degrade cyclic di-GMP in cells (Römling et al., 2013; Valentini and Filloux, 2016). At least 40 such proteins directly synthesize and/or degrade cyclic

TABLE 2 | Key polymeric substances in *P. aeruginosa* biofilm formation and development.

Name	Identity/Chemistry	Precursor(s)	Biosynthesis	Property	References
Psl	Exopolysaccharide / Repeating pentasaccharide containing D-mannose, D-glucose and L-rhamnose	GDP-D-mannose, UDP-D-glucose and dTDP-L-rhamnose	*The pslA-O operon*	Neutral charge	Byrd et al., 2009; Colvin et al., 2012
Pel	Exopolysaccharide/ Partially acetylated (1→4) glycosidic linkages of N-acetylgalactosamine and N-acetylglucosamine.	UDP-sugar nucleotide/ uncharacterized	The *pelA-G* operon	Positively charged	Franklin et al., 2011; Jennings et al., 2015
Alginate	Exopolysaccharide/ O-acetylated 1–4 linked D-mannuronic acid and variable proportions of its 5-epimer L-guluronic acid	GDP-mannuronic acid	The alginate operon (*algD, alg8, alg44, algK, algE, algG,algX, algL, algI, algJ, algF*, and *algA) and algC*	Negatively charged	Hay et al., 2010; Moradali et al., 2015
eDNA	Nucleic acid	–	Cell lysis	Negatively charged	Allesen-Holm et al., 2006; Ma et al., 2009
Type 4* pili	Multiprotein complex/ Type 4a pili	–	The *pilM/N/O/P/Q* and the *fimU-pilVWXY1Y2E* operons	–	Ayers et al., 2009; Burrows, 2012
Flagella*	Multiprotein complex	–	At least 41 genes clustered in three regions of the genome encode flagellin structural and regulatory components	–	Jyot and Ramphal, 2008

** Are not commonly considered as classical matrix molecules of biofilm, but important for biofilm maturation.*

di-GMP in *P. aeruginosa* which controls cellular level of this molecule in response to perceived stimuli (Ryan et al., 2006). The second group is represented by cyclic di-GMP sensing proteins which also act directly as effectors or via protein-protein interactions to mediate the output response (Römling et al., 2013). For example, cyclic di-GMP is essential for the activation of alginate polysaccharide polymerization (Remminghorst and Rehm, 2006). Experimental evidence indicated that a pool of cyclic di-GMP is synthesized by MucR (a hybrid GGDEF/EAL domain-containing protein) in the proximity of the alginate biosynthesis/secretion multi-protein complex of *P. aeruginosa* (Hay et al., 2009b; Wang et al., 2015). Cyclic di-GMP binds to PilZ domain of Alg44 protein and allosterically activates alginate polymerization via interaction with Alg8 glycosyltransferase (Hay et al., 2009b; Moradali et al., 2015; Wang et al., 2015). Also, cyclic di-GMP binding to FleQ, a transcriptional master regulator represses flagella biosynthesis while it concomitantly derepresses the expression of *pel* and *psl* genes (Baraquet et al., 2012; **Figure 2**). Likewise, there are many other receptor/effector proteins which enhance required pathways for biofilm formation upon cyclic di-GMP binding while they inhibit motility and other virulence factor synthesis pathways.

In addition, the Wsp chemosensory system in *P. aeruginosa* is homologous to chemotaxis signaling pathways which regulate cyclic di-GMP synthesis via signal transduction (**Figure 2**). A cascade of phosphorylations is triggered upon surface attachment and possibly sensing mechanical stress or other environmental stimuli which then activate the cyclic di-GMP synthesizing protein WspR promoting biofilm formation (Hickman et al., 2005; Porter et al., 2011).

Furthermore, transduction of phosphorylation events via two-component regulatory systems controls biofilm formation in a stage-specific manner (**Figure 2**). This network consists of BfiRS, BfmRS, and MifRS and GacS/GacA regulatory components (Petrova and Sauer, 2009). The GacS/GacA two-component system is part of the global regulatory pathway comprising LadS/RetS/GacS/GacA/RsmA proteins (**Figure 2**). This pathway controls many physiological responses at post-transcriptional level and is involved in both motility-sessility and acute-chronic infection transitions. Of this regulatory pathway, the RNA-binding protein RsmA negatively controls biofilm formation pathways while it induces production of T3SS, type 4 pili and other virulence factors. RsmA binds to *psl* mRNA and inhibits the translation of required proteins for Psl polysaccharide biosynthesis (Irie et al., 2010; Jimenez et al., 2012). It also represses production of GGDEF/EAL encoding proteins; hence, it inhibits elevation of cyclic di-GMP levels. In *P. aeruginosa*, under stress conditions, this pathway generates non-coding RNAs (ncRNAs) known as RsmY and RsmZ which counteract RsmA translational repression activity, consequently derepressing biofilm formation mainly via cyclic di-GMP level increase resulting in exopolysaccharides production (Jimenez et al., 2012).

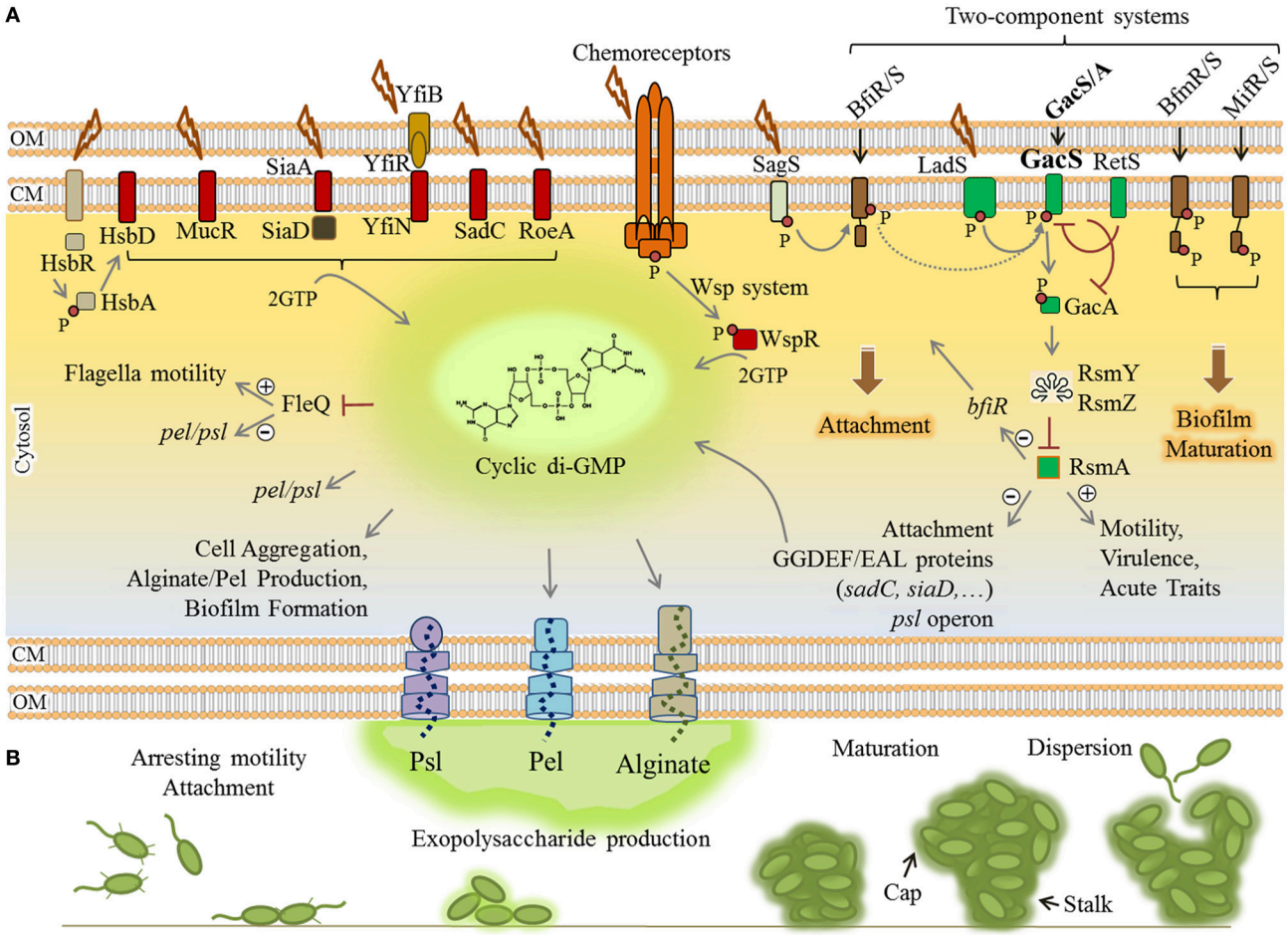


FIGURE 2 | Regulatory networks underlying biofilm formation by *P. aeruginosa*. (A) Elevation of the cyclic di-GMP molecule is a key determinant for the motility-sessility switch. Environmental cues are sensed by various proteins localized in the envelope of the cells where these proteins contribute to two-component systems (brown/green rectangles), chemoreceptor-like system (orange complex) and other receptor mediated signaling pathways (arranged in the left side of figure). Either triggered as phosphorylation cascades (small red circle) or protein-protein interactions, the signals induce diguanylate cyclases (containing GGDEF motif) (red rectangles) to synthesize cyclic di-GMP from two molecules of GTP (guanosine-5′-triphosphate). Consequently, cyclic di-GMP sensing proteins act as receptor/effector for specific outputs such as induction of alginate and Pel polymerization, inhibition of motility and derepression of *psl/pel* expression via FleQ, induction of attachment and biofilm formation/maturation triggered by two component systems. The two-component systems are interconnected and the LadS/RetS/GacS/GacA/RsmA regulatory network (green rectangles) plays a key role in the phenotypic switch from motility to sessility and downregulation of QS and virulence factor production. **(B)** Various stages of biofilm formation and development were represented. Plus and minus signs represent positive and negative effect of transcriptional regulators, respectively. CM, cytoplasmic membrane; OM, outer membrane.

There are other regulatory pathways known to be involved in cyclic di-GMP turnover in response to external stimuli, but the further precise function still remains to be elucidated (**Figure 2**).

The Role of QS in Biofilm Development and Maturation

In addition to abovementioned regulatory networks, biofilm residents utilize QS systems for cell-to-cell communication and spatio-temporal regulation of expression of specific genes. During chronic infection, a major proportion of the colonizing population was thought to lose QS due to hypermutation events and phenotypic alterations. However, further investigations have now revealed that genes involved in the progress of biofilm maturation and persistence are positively regulated by QS in *P. aeruginosa*. Indeed QS-deficient mutants of *P. aeruginosa* (i.e., Δ*lasR*Δ*rhlR* and Δ*lasI*Δ*rhlI*) formed thin and much less developed biofilms which were more sensitive to antibiotic treatments and eradication (Shih and Huang, 2002; Nelson et al., 2009). Furthermore, Bjarnsholt et al. (2010) demonstrated that at least a part of QS pathways i.e., *rhl* encoded system and the production of C4-HSL signals was retained in predominantly mucoid population at the end of chronic stages coinciding with overproduction of alginate and rhamnolipids (Bjarnsholt et al., 2010). The biosurfactants, rhamnolipids, have been suggested to play an active role in maintenance of the biofilm architecture by contributing to the formation of internal cavities within the mature biofilm, allowing proper flow of water and nutrients (Davey et al., 2003; Boles et al., 2005; Dusane et al., 2010;

Chrzanowski et al., 2012). Additionally, the production of pel polysaccharide, eDNA and QS-controlled production of pyocyanin are critical for biofilm maturation. Pel cross-links eDNA in the biofilm stalk via ionic interactions and it serves as important structural components of the biofilm matrix of *P. aeruginosa* (Jennings et al., 2015). Furthermore, pyocyanin molecules can promote eDNA release by inducing bacterial cell lysis. Pyocyanin binds to eDNA increasing its solution viscosity which influences the physicochemical interactions of the biofilm matrix with environment as well as facilitates cellular aggregations (Das et al., 2013, 2015). Collectively, such molecular and cellular interactions in combination with other polymeric substances lead to establishment of a robust and mature biofilm.

ANTIBIOTIC RESISTANCE MECHANISMS

Indeed, the emergence of antibiotic resistant bacteria is a global health issue. Among identified notoriously multi-drug resistant (MDR) bacteria, *P. aeruginosa* has been introduced as a major concern with a growing threat to global health resulting in dramatically increasing prevalence of nosocomial and chronic infections. This is due to the extraordinary capacity of these bacteria to develop resistance against a wide range of antimicrobials through various molecular mechanisms which are often simultaneously present in clinical isolates. Although each resistance mechanism is related to a specific class of antibiotics, multiple mechanisms mediate variably resistance to each class of antibiotics (Potron et al., 2015). Furthermore, the contribution of each mechanism varies from country to country. Loss or reduced copy numbers of OprD and overproduction of active efflux pumps, AmpC β-lactamase and extended-spectrum β-lactamases have been mainly reported as main contributors to multi-drug resistance phenotypes of *P. aeruginosa* isolates.

Recent reviews have described the prevalence and contribution of each resistance mechanism to each class of antibiotics in detail (Lister et al., 2009; Strateva and Yordanov, 2009; Sun et al., 2014; Hong et al., 2015; Potron et al., 2015). Here, we reviewed the most frequent and well-understood findings which are classified into intrinsic, acquired and adaptive mechanisms, and we provide an update on our understanding of how *P. aeruginosa* can survive antibiotic treatments.

Intrinsic Resistance Mechanisms

Like many Gram-negative bacteria, *P. aeruginosa* can be intrinsically resistant to particular antibiotics. Such intrinsic resistance mechanisms stem from the existence of genes in bacterial genome encoding inherent properties of cell structures and composition providing protection against toxic molecules and antimicrobials. It can also be contributed by the lack of susceptible sites which naturally exist in antibiotic sensitive species (e.g., resistance to triclosan) (Lambert et al., 2011; Blair et al., 2015; **Figure 3**).

However, hydrophilic antibiotics can enter cells by diffusing through membrane channels or porin proteins in a non-specific manner. As one of the intrinsic mechanisms, *P. aeruginosa* limits antibiotic entry by reducing the number of non-specific porin proteins and replacing them with specific or more-selective channels for taking up required nutrients resulting in lowered permeability to toxic chemicals (Tamber and Hancock, 2003; **Figure 3**). *P. aeruginosa* resistance to currently used broad-spectrum drugs such as carbapenems and cephalosporins is commonly caused by this adaptation (El Amin et al., 2005; Baumgart et al., 2010). Many of the clinical strains of *P. aeruginosa* displaying resistance to carbapenems such as imipenem are deficient in the OprD porin which specifically facilitates the diffusion of basic amino acids, small peptides as well as carbapenems into the cell (Trias and Nikaido, 1990; Strateva and Yordanov, 2009).

Active multidrug efflux pumps greatly contribute to antibiotic resistance observed in *P. aeruginosa*. The involved genes are ubiquitous in Gram-negative bacteria and they are located on the genome or plasmids. The multidrug efflux pumps are multi-protein complexes spanning the envelope of Gram-negative bacteria. They are responsible for expelling various toxic materials and a wide range of antimicrobials. Because of their broad substrate specificities they display resistance against different classes of antibiotics which are chemically unrelated (Blair et al., 2015; Venter et al., 2015).

P. aeruginosa possesses four well known active multidrug efflux pumps including MexAB-OprM, MexXY/OprM(OprA), MexCD-OprJ, and MexEF-OprN (**Figure 3**). The gene sets encoding these systems are under different regulatory factors; therefore, the expression levels of these systems significantly differ under various conditions. The MexAB-OprM and MexXY/OprM(OprA) are the most clinically important sets due to their large prevalence in clinical strains and significant contribution to a wide range of antibiotics (Avrain et al., 2013). The *mexAB-oprM* genes show a stable and constitutive expression in the cell guaranteeing a protective basal level production of the MexAB-OprM system to consistently expel a wide range of toxic molecules and antibiotics (Li et al., 2015). Hence it mainly contributes to natural resistance to antibiotics. The *mexXY-(oprA)* genes show lower basal expression levels and are mainly induced in response to protein synthesis inhibitors that target the ribosomal machinery (Matsuo et al., 2004; Hay T. et al., 2013). Both *mexCD-oprJ* and *mexEF-oprN* genes are not typically expressed in wild-type strains or their expression is very low and they have been proposed not to contribute significantly to natural antibiotic resistance (Llanes et al., 2011; Li et al., 2015).

There are other forms of multidrug efflux pumps such as MexJK, MexGHI-OpmD, MexVW, MexPQ-OpmE, MexMN, and TriABC. They are not expressed in wild-type strains but may contribute to adaptive resistance against antibiotic or biocide agents when expressed in resistant strains (Lister et al., 2009; Avrain et al., 2013).

On the other hand, they might play role in other physiological pathways as well. For example, The MexEF-OprN and MexGHI-OpmD sets can modulate QS systems by exporting the quinolone signaling molecule PQS reducing its cellular concentration resulting in the reduction of virulence factor production, which is presumably in favor of establishment of chronic infections (Köhler et al., 2001; Aendekerk et al., 2005; Lamarche and Déziel, 2011). However, many of these mechanisms remain still

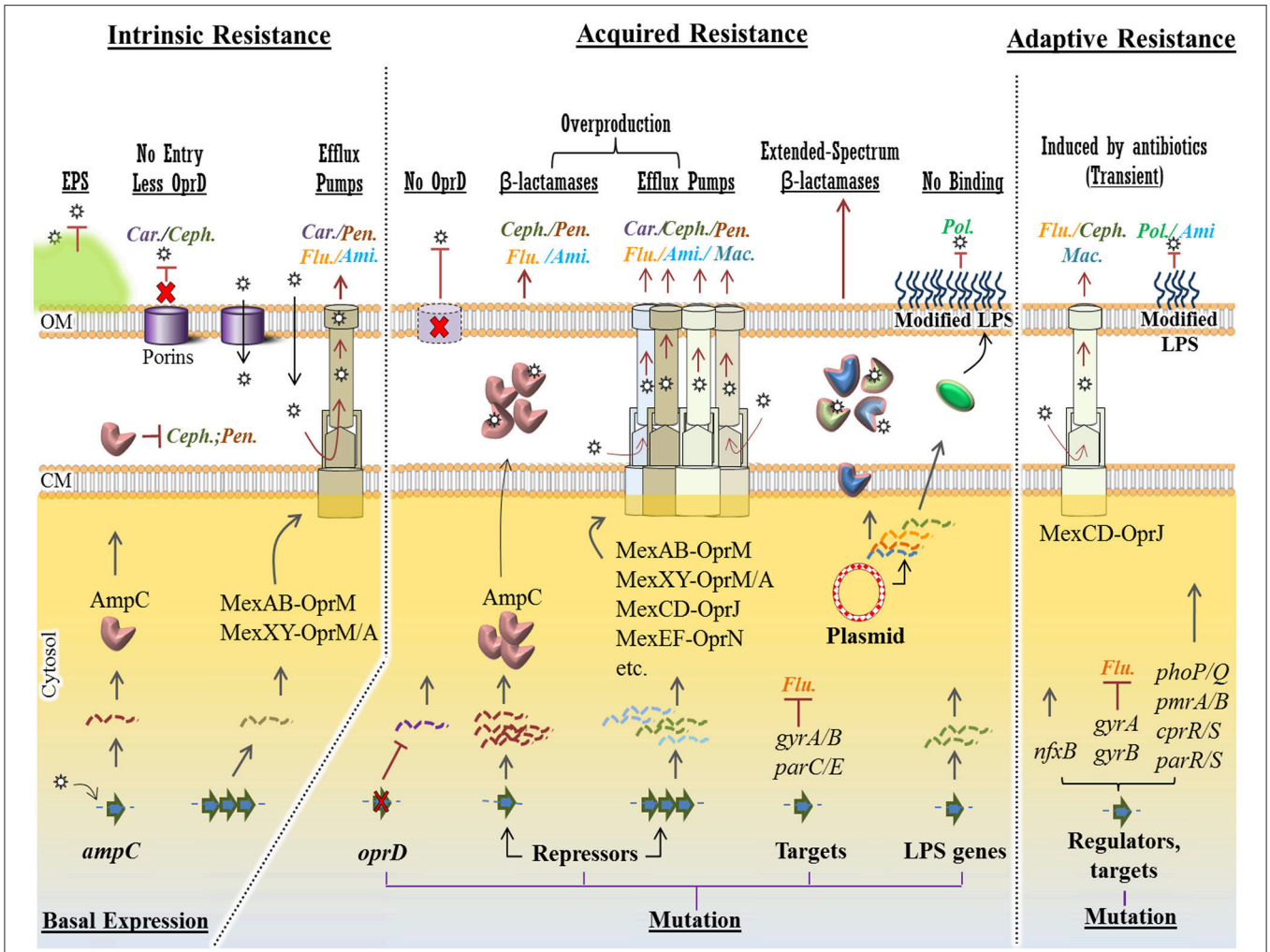


FIGURE 3 | Intrinsic, acquired and adaptive mechanisms confer antibiotic resistance in *P. aeruginosa*. For each mechanism, various molecular strategies, which confer resistance to specific class of antipseudomonal antibiotics (Car., Carbapenems; Ceph., Cephalosporins; Pen., Penicillins; Ami., Aminoglycosides; Flu., Fluoroquinolones; Mac., Macrolides and Pol., Polymyxins), were presented at the top of the figure (underlined) Intrinsic mechanisms such as structural barriers [e.g., EPS (extracellular polymeric substances)], OprD reduction and basal production of AmpC β-lactamase and MexAB/XY efflux pumps confer a basal resistance to some group of antibiotics. However, in acquired resistance, mutational changes in the *oprD* gene, transcriptional repressors causingupregulation of resistance genes and efflux pumps conferring resistance against a wider spectrum of antibiotics. Plasmid-mediated resistance is very potent as a variety of resistance genes can be exchanged among bacteria. Either mediated by mutational changes in the genome or in plasmids, resistance to polymyxins occurs via modification of LPS (lipopolysaccharide) components hindering binding of the antibiotic to this layer. Adaptive resistance occurs in the presence of antibiotics mainly via mutation in regulatory genes. This is a transient and reversible resistance, which will reverse upon removal of antibiotics. Stars represent antibiotics and dashed/wavy lines represent transcriptional levels of each gene product. CM, cytoplasmic membrane; OM, outer membrane.

unclear with regard to their connection with other physiological pathways and their clinical relevance.

Another player of intrinsic resistance and basal lower level antibiotic susceptibility in *P. aeruginosa* is the gene encoding an inducible β-lactamase (AmpC) (**Figure 3**). Particularly, chromosomal expression and production of AmpC confers low level resistance to aminopenicillins and most cephalosporins because these antibiotics strongly induce the production of AmpC which consequently hydrolyzes these substrates (Oliver et al., 2015). However, through adaptive or acquired resistance mechanisms AmpC can be overproduced, consequently conferring resistance to a wider range of antibiotics such as aminoglycosides and fluoroquinolones (Umadevi et al., 2011). These mechanisms will be further discussed later.

Acquired Resistance Mechanisms

P. aeruginosa can acquire resistance to antibiotics through mutation of intrinsic genes or horizontal acquisition from other bacteria through transferring plasmids carrying genetic materials encoding for antibiotic resistance (Davies, 1997; Davies and Davies, 2010). Contrary to intrinsic mechanisms, acquired resistance is related to antibiotic selection and this selective advantage occurs in the presence of antibiotic compounds leading to irreversible resistant population (Lee et al., 2016).

Therefore, similar to intrinsic resistance, acquired resistance is stable too and it can be transmitted to progeny.

However, due to over-expression of resistance genes and transmissibility by plasmids, acquired resistance is a potent mechanism which confers resistance to a wide spectrum of antibiotics as well as leads to increased prevalence among clinical and environmental strains.

Boosted Antibiotic Resistance via Mutations

Intrinsic resistance genes are negatively or positively regulated by one or more regulatory mechanisms which confer a basal lower susceptibility of *P. aeruginosa* to a narrow spectrum of antibiotics. However, mutation in regulatory pathway could increase promoter activities resulting in unleashing gene expression and overproduction of protein products such as AmpC and multi-drug efflux pumps systems. Consequently, it causes higher level of resistance to antibiotics (Blair et al., 2015; **Figure 3**).

As a common mutational feature of *P. aeruginosa* isolates, resistant clinical mutants display a constitutive high level of AmpC production even in the absence of antibiotic inducers. This is mainly due to mutational inactivation of *ampD* (repressor of *ampC*) and specific point mutations of *ampR*, both encoding two regulatory proteins important in induction of the *ampC* gene (Juan et al., 2005; **Figure 3**). Consequently, it turns into a major cause of greater resistance to a wide range of antibiotics such as most of the β-lactams (e.g., ticarcillin and piperacillin) as well as monobactams, third-generation and fourth-generation cephalosporins (Lister et al., 2009; Berrazeg et al., 2015). One study showed that 73% of tested clinical strains showed AmpC overproduction (Henrichfreise et al., 2007).

Several regulatory loci such as *mexR*, *nalD, nalB,* and *nalC* negatively control the expression of the *mexAB-oprM* operon in *P. aeruginosa*. On the other hand, various loss-of-function mutations in these loci derepress the expression of the *mexAB-oprM* operon leading to the overproduction of MexAB-OprM complex conferring a greater resistance to carbapenem antibiotics (Quale et al., 2006; Lister et al., 2009; Kao et al., 2016; **Figure 3**). Likewise, overproduction of other multidrug efflux pumps such as MexXY and MexCD-OprJ can occur via mutations in regulatory loci leading to unleashing gene expression and a greater resistance to a variety of antimicrobial agents (Lister et al., 2009; **Figure 3**).

Another clinically important and prevalent mutational alteration is attributed to OprD porin channel. This porin channel is localized in the outer membrane of *P. aeruginosa* and it is characterized as a carbapenem-specific porin (**Figure 3**). Therefore, loss or reduction of OprD can reduce permeability of the outer membrane to carbapenems (Epp et al., 2001; Gutiérrez et al., 2007; Kao et al., 2016). The emergence of resistance to imipenem and reduced susceptibility to meropenem has been reported upon the occurrence of *oprD* mutations. Genetic alteration in *oprD* can occur via nucleotide insertion or deletion and point mutations resulting in frameshift of the gene sequence, amino acid substitution, shortened putative loop L7 and premature stop codons (Kao et al., 2016). Furthermore, downregulation of *oprD* expression can be mediated by other regulatory factors such as MexT which itself concurrently upregulates *mexEF-oprN* expression (Köhler et al., 1997; Ochs et al., 1999).

Additionally, fluoroquinolone resistance among *P. aeruginosa* isolates can be mediated by either mutational changes within the fluoroquinolone targets i.e., DNA gyrase (*gyrA* and *gyrB*) and/or topoisomerase IV (*parC* and *parE*) or overproduction of active or inducible efflux pumps (Lee et al., 2005; Sun et al., 2014; **Figure 3**).

Plasmid-Mediated Resistance

Bacterial plasmids serve a central role as a potent vehicle for acquiring resistance genes and subsequent delivery to recipient host. This is so-called horizontal gene transfer whereby genetic elements can be transferred between bacterial cells particularly via conjugation. Some resistance plasmids are broad host range which can be transferred among various species via bacterial conjugation, while narrow host range plasmids are transferred among a small number of cells from similar bacterial species. For example, plasmid RP1 can transfer resistance genes to most Gram-negative bacteria (Kenward et al., 1978).

Plasmid-encoded antibiotic resistance confers resistance to different classes of antibiotics that are currently applied in frontline of clinical treatments such as β-lactams, fluoroquinolones and aminoglycosides (Bennett, 2008; **Figure 3**). So far, *P. aeruginosa* resistance via horizontal gene transfer has been reported for the genes encoding β-lactam-hydrolyzing enzymes known as the extended-spectrum β-lactamases and the carbapenemases, aminoglycoside-modifying enzymes, 16S rRNA methylases resulting in high-level pan-aminoglycoside resistance (Poole, 2011).

The genes encoding extended-spectrum β-lactamases and carbapenemase are clinically important not only due to their hydrolyzing activity on a wide range of β-lactams such as carbapenems and extended-spectrum cephalosporins, but also for their worldwide prevalence (Paterson and Bonomo, 2005; Blair et al., 2015; Sullivan et al., 2016). The global epidemiology of carbapenem-resistant *P. aeruginosa* was recently analyzed by Hong et al (Hong et al., 2015). They reported that the geographical prevalence of these genes differs from country to country, whereas the genes encoding carbapenemases such as IMP, VIM, and NDM type metallo-β-lactamases have been found in all continents (Johnson and Woodford, 2013; Meletis and Bagkeri, 2013; Hong et al., 2015). Almost all types of transferable carbapenemases, except SIM-1, have been detected in *P. aeruginosa*, and the prevalence of carbapenem-resistant isolates of *P. aeruginosa* is gradually increasing (Meletis and Bagkeri, 2013; Hong et al., 2015).

It is of concern that transferable plasmids carrying some of the resistance genes are mobile among a wide range of unrelated Gram-negative bacteria which increases the antimicrobial resistance transfer rate causing increasing treatment complications (Hong et al., 2015). Recent findings about antibiotic resistance have been even more concerning and warning. Liu et al. reported the first evidence of

plasmid-mediated colistin resistance from China (Liu et al., 2016; **Figure 3**). Colistin (or polymyxin E) belongs to the family of polymyxins. The members of this class of antibiotics such as polymyxin B and colistin have been the last resort for antibiotic treatment of carbapenem-resistant bacteria such as *P. aeruginosa* isolates and Enterobacteriaceae (Falagas and Kasiakou, 2005). Resistance to polymyxins was previously reported to occur via chromosomal mutations (Moskowitz et al., 2012; Gutu et al., 2013), however, new evidence suggests plasmid-mediated resistance through the mobilization of the *mcr-1* gene which consequently confers resistance to colistin (**Figure 3**). This gene was discovered in *E. coli* strain SHP45 collected from agricultural products. It is more concerning that the plasmid carrying *mcr-1* was mobilized into *K. pneumoniae* and *P. aeruginosa* via conjugation (Liu et al., 2016). This finding has triggered serious concerns about the emergence of pan-drug-resistant Gram-negative bacteria leading to almost untreatable infections. Recent findings provided some evidence of the spreading high-risk of clone ST654 of *P. aeruginosa* containing the genomic $bla_{\mathrm{NDM-1}}$ resistance gene which also conferred resistance to colistin. It is likely that $bla_{\mathrm{NDM-1}}$ was acquired via genetic exchange between *P. aeruginosa* and *K. pneumoniae* isolate in the same patient (Mataseje et al., 2016).

Adaptive Resistance Mechanisms

Compared to other types of resistance mechanisms, adaptive mechanisms are not really well understood. Adaptive resistance is an unstable and transient form of resistance, which is induced in the presence of specific antibiotics and other environmental stresses. This type of resistance mainly relies on induced alterations in gene expression and protein production or alterations in antibiotic targets and it is reversal upon removal of external stimuli leading to re-gaining susceptibility (Barclay et al., 1992; Xiong et al., 1996; Fernández et al., 2011). This mechanism has been seen mediating the resistance of *P. aeruginosa* isolates to β-lactams, aminoglycosides, polymyxins and fluoroquinolones (Zhang et al., 2001; Poole, 2005; Fernández et al., 2010; Khaledi et al., 2016).

It has been seen that once strains encounters certain concentrations of antibiotics, they can tolerate higher concentrations in subsequent exposures, while cross-resistance to other antibiotics may occur as well (Mouneimné et al., 1999; Fujimura et al., 2009; Fernández et al., 2011; Pagedar et al., 2011). Furthermore, these alterations may link to other physiological events triggered by other stimuli and stresses as well as mutations in some specific genes (Xiong et al., 1996; Karlowsky et al., 1997; Fernández et al., 2011).

Using isolates from CF patients, it was shown that adaptive resistance of *P. aeruginosa* to fluoroquinolones such as ciprofloxacin is due to multiple mutations in the known-resistance genes including the *gyrA, gyrB, nfxB,* and *orfN* which were concomitant with mutations in the genes involved in cyclic di-GMP signaling (**Figure 3**). Mutations of *nfxB* were prevalent leading to loss of function of NfxB transcriptional repressor and consequently leading to the overproduction of MexCD-OprJ efflux pump (Wong et al., 2012; **Figure 3**). This efflux pump is an important determinant of resistance to fluoroquinolone antibiotics (Hirai et al., 1987). On the other hand, another study showed that expression of the *mexCD-oprJ* genes depends on the sigma factor AlgU and leads to resistance to the biocide chlorhexidine (Fraud et al., 2008). AlgU is well-known stress response sigma factor which positively regulates overproduction of alginate in mucoid isolates (Hershberger et al., 1995).

Another group showed that *P. aeruginosa* can acquire and lose resistance in the presence and absence of colistin, respectively. This occurred via adaptive multiple mutational mechanisms and genetic reversion (Lee et al., 2016). It was also demonstrated that resistance to certain polycationic antimicrobials such as aminoglycosides, polymyxins and cationic antimicrobial peptides can be mediated by altering the lipid A structure in LPS. This was caused by multiple mutations in cognate regulatory proteins such as the two-component systems PhoP-PhoQ, PmrA-PmrB, CprR-CprS, and ParR-ParS (Barrow and Kwon, 2009; Fernández et al., 2012; **Figure 3**). Other studies showed that further and complex genetic alterations affecting regulatory pathways including those causing amino acid substitutions in these cognate regulatory proteins such as PhoQ and PmrB are involved in polymyxin resistance. This is why the mechanism of resistance of *P. aeruginosa* to colistin was found to vary among isolates (Lee et al., 2016). Interestingly, this study showed that the acquisition of colistin resistance via many amino acid substitutions is reversible in colistin-susceptible revertants. However, even in the absence of colistin, resistance was preserved for some time and emergence of revertants may not occur so fast (Lee et al., 2016).

QS-Dependent Antibiotic Resistance

Some direct and indirect evidences have been found linking the QS systems with antibiotic resistance mechanisms in *P. aeruginosa* (Rasamiravaka and El Jaziri, 2016), but further exploration is needed for better understanding. Using clinical strains of *P. aeruginosa*, it was shown that the *las* system positively links to the expression of *mexY* gene encoding the inner-membrane drug/H^+ antiporter protein MexY (Pourmand et al., 2015) which is a key subunit of the MexXY-oprM complex known as a major determinant of aminoglycoside resistance (Lau et al., 2014). On the other hand, some studies showed that CF-infecting strains with the common *lasR* loss-of-function mutations were more resistant to therapeutic antibiotics such as tobramycin, ciprofloxacin and ceftazidime. The reported antibiotic resistances in the *lasR* mutants were attributed to increased β-lactamase activity, bacterial metabolic adaptation or metabolic shifts (D'Argenio et al., 2007; Hoffman et al., 2010). However, the relationship of antibiotics susceptibility with the *rhl* encoded QS system and production of C4-HSL signals remains unclear (Bjarnsholt et al., 2010). Some supporting evidence was obtained by, treating *P. aeruginosa* biofilms with ciprofloxacin which upregulated the production and secretion of the virulent factor LasB, which is under the control of Rhl QS system (Oldak and Trafny, 2005; **Figure 1**).

Furthermore, two independent studies reported that the clinical strains of *P. aeruginosa* with QS-deficient phenotypes and negative for the production of QS-dependent virulence factors could cause infections and tend to be less susceptible to antimicrobial agents (Karatuna and Yagci, 2010; Wang

H. et al., 2013). However, it was not shown how these mechanisms might link to each other while many of these clinical strains could also form biofilms with antibiotic resistance traits and many regulatory pathways for biofilm development are under the control of QS systems. Zhao et al. reported some supporting information showing the importance of QS systems in both biofilm formation and antimicrobials induced expression of *ampC* (Zhao et al., 2015). Earlier studies showed that by overexpressing the chromosomal type 1 β-lactamase, QS-dependent virulence factors were reduced and strains were less virulent (Ramisse et al., 2000). Also, Kong et al. analyzed the dual role of the AmpR transcriptional regulator where it positively regulated β-lactamases and negatively regulated the virulence factors through QS systems (Kong et al., 2005).

Balasubramanian et al. analyzed co-regulatory and transcriptional networks of three co-existing mechanisms involved in β-lactam resistance, alginate production and modulation of virulence factor expression. They showed that while AmpR positively and negatively regulates β-lactamases and QS-dependent proteases, respectively, there is an intimate crosstalk between the AmpR regulon and the master regulator AlgU which positively regulates alginate production (Balasubramanian et al., 2011). This gave more insight into the complexity of such co-existing networks. Recent findings also showed that high levels of cyclic di-GMP mediated by the SagS regulator contributes to elevated antibiotic resistance via BrlR regulon-dependent upregulation of cognate genes encoding MexAB-OprM and MexEF-OprN multidrug efflux pumps (Gupta et al., 2014).

The periplasmic TpbA tyrosine phosphatase was also reported as a regulatory candidate for linking QS signaling and biofilm formation. This protein was shown to be positively regulated by the *las* QS system at transcriptional level. Upon production of TpbA and its phosphatase activity in the periplasm, the cyclic di-GMP synthesizing protein TpbB is dephosphorylated at a tyrosine residue in periplasmic domain leading to inactivation of TpbB and a reduction in cyclic di-GMP levels and in turn Pel production, hence inhibiting biofilm development (Ueda and Wood, 2009). TpbA-dependent cyclic di-GMP reduction was also linked to increasing eDNA release by cell lysis (Ueda and Wood, 2010).

Overall, the reason for inconclusive information about the relation of the QS system and antibiotic resistance mechanisms is based on the fact that there are various layers of regulatory pathways associated with both QS systems and antibiotic resistance mechanisms in *P. aeruginosa*. Therefore, understanding the interplay between hierarchical QS systems and various antibiotic resistance mechanisms needs further exploration.

ADAPTIVE RADIATION FOR PERSISTENCE

Adaptation to the surrounding environments is an extraordinary capability of *P. aeruginosa*. It enables *P. aeruginosa* to inhabit diverse ecological niches such as colonization of various hosts as well as long term persisting infections. The adaptation process is designated as adaptive radiation by which initial clones would diversify into a variety of genotypes and phenotypes over time until the most favorable and adapted descendants are selected for long term persistence (Hogardt and Heesemann, 2010). A typical example is adaptation of *P. aeruginosa* isolates to the CF airways. Various studies have shown that initial colonization of CF lungs is caused by wild-type strains existing in the environment. For bacteria, the CF lungs encompass various stresses such as oxidative stresses and immune responses and inter-species competition followed by antibiotic treatment. Therefore, initial clones undergo substantial adaptation processes to survive such hostile environments.

Here, adaptive radiation is mainly due to intense genetic adaptations leading to the thousands of generations displaying diverse genotypes and phenotypes that emerge *in vivo*, while subjected to selection pressure imposed by the CF lung milieu (**Figure 4**). Therefore, selected variants display different genotypes when compared with initial wild-type colonizers and persist in the CF lungs leading to clonal expansion within patients and establishment of chronic infections (Mathee et al., 1999; Kong et al., 2005; Boles and Singh, 2008; Driffield et al., 2008; Workentine et al., 2013). By assessing a wide selection of phenotypes, Workentine et al. showed that the overall population structure in one chronically infected patient can be much more heterogeneous in phenotypes than what has been previously documented (Workentine et al., 2013). Furthermore, it has been reported that transmission of strains from patient to patient can result in the coexistence of highly divergent bacterial lineages (Winstanley et al., 2016).

Generally, phenotypic adaptation of these strains include slow growth, auxotrophy, virulence deficiency via downregulation of QS systems, loss of motility, biofilm formation, alginate overproduction and mucoid phenotype, antibiotic resistance, hypermutability, and lipopolysaccharide modifications. Downregulation of virulence factors such as flagella motility, T2SS/T3SS apparatus, and toxic components results in less inflammatory and phagocytic responses since the pathogen is less detectable for the immune system (Mahenthiralingam et al., 1994; Hogardt and Heesemann, 2010). Analysis of many clinical isolates showed these alterations represent convergent molecular evolution among many clinical isolates and mutation of 52 genes are mainly responsible for substantial phenotypic alterations associated with virulence traits and resistance (Diaz Caballero et al., 2015; Marvig et al., 2015). Of these genes, common adaptive mutations occur in regulatory genes including *lasR, pvdS, rpoN, mucA, mexT, nfxB, mexR, nalD, retS, and ampR* (**Figure 4**). Collectively, this leads to remodeling regulatory networks and developing a general adaptation pattern as explained below (Higgins et al., 2003; Hogardt and Heesemann, 2010; Rau et al., 2010; Winstanley et al., 2016).

Mutation of *lasR*, *pvdS*, and *rpoN* impairs central QS system signal processing leading to the deficiency in virulence traits. In wild-type strains, the LasR and PvdS regulators control the expression of a large number of genes including key virulence factors (**Table 1**, **Figure 4**) and pyoverdine for iron acquisition, respectively (**Table 1**; Hoffman et al., 2009; Imperi et al., 2010; Jiricny et al., 2014; LaFayette et al.,

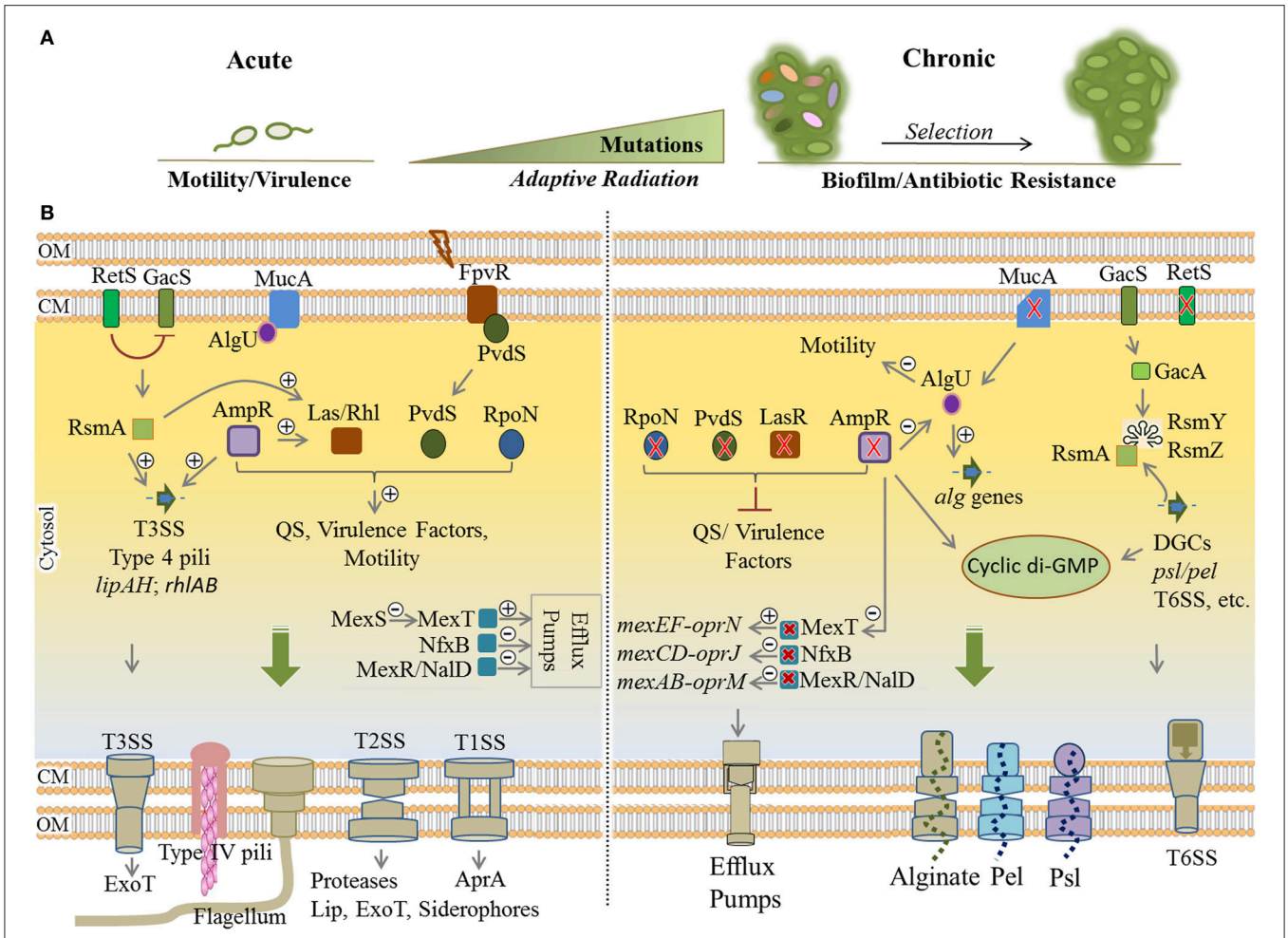


FIGURE 4 | Remodeling of regulatory networks in *P. aeruginosa* during adaptive radiation and transition from acute to chronic infections. (A) During pathogenesis, adaptation to the CF lung environment occurs through "adaptive radiation" where intense genetic mutations lead to diverse genotypes and phenotypes (colorful ellipsoids) within bacterial populations followed by the selection of colonizers. Mutational adaptation and selection of generations drive bacterial transition from acute to chronic traits. **(B)** Remodeling of key regulatory networks between acute and chronic infections occurs mainly via mutational adaptation in cognate genes. Mutated *lasR*, *ampR,* and *retS* genes are key determinants in this process by which QS, virulence factor production and motility are downregulated, while synthesis of cyclic di-GMP, exopolysaccharides and various multidrug efflux pumps are upregulated. Mutation of *mucA* results in a defect in MucA (anti-sigma factor) releasing AlgU (positive regulator of alginate operon) that induces overproduction of alginate and the mucoid phenotype. Of important acute traits are flagellum, type 4 pili, T1SS, T2SS, T3SS (types 1 to 3 secretion systems), ExoT (exotoxins), Lip (lipases), AprA (alkaline proteases). The type 6 secretion system (biofilm-associated and toxin-delivering device to other bacteria) and efflux pumps and the production of EPS are part of chronic traits which confer antibiotic resistance and/or mediate biofilm formation. Plus and minus signs represent positive and negative effect of transcriptional regulators, respectively. Red cross indicates mutagenesis. CM, cytoplasmic membrane; OM, outer membrane.

2015). The alternative sigma factor RpoN has been also found to regulate many cell functions such as motility and virulence factors production via QS system (Cai et al., 2015; **Figure 4**). Additionally, knocking out mutation of *mucA* locus encoding anti-sigma factor MucA results in releasing the RNA polymerase sigma factor σ^{22} (AlgU) which itself positively regulates expression of the alginate biosynthesis operon and stress response mechanisms while it negatively regulates several virulence factors such as flagella, pili, T3SS, and Rhl quorum sensing signals (Folkesson et al., 2012). Development of the mucoid phenotype mediated by alginate overproduction as well as the formation of highly structured biofilms is the hallmark of chronic infections (Schurr et al., 1996; Hay I. D. et al., 2013; **Figure 4**).

Another common mutation has been reported in the *mexT* locus resulting in activation of MexT, the positive regulator of MexEF-OprN efflux pump, which in turn led to antibiotic resistance. In addition, the induction of MexEF-oprN production is linked to extruding QS signaling molecules and reduction of virulence factor production (Tian et al., 2009; **Figure 4**). The MexEF-OprN production is undetectable in wild-type strains due to the non-functionality of the *mexT* gene (Maseda et al., 2000, 2010). Furthermore, mutations of repressor genes *nfxB* and *mexR/nalD* in clinical strains upregulated the production of

the MexCD-OprJ and MexAB-OprM efflux pumps, respectively, conferring resistance to a wider range of antibiotics (Higgins et al., 2003; Sobel et al., 2005; Jeannot et al., 2008; Rau et al., 2010; **Figure 4**). Also, mutation of other genes such as *gyrA*/*gyrB* (DNA gyrase), *mexZ* (transcriptional regulator of the *mexXY*) and *mexS* (transcriptional regulator of the *mexEF*) are commonly attributed to antibiotic resistance during mutational adaptations (Marvig et al., 2015; **Figure 4**).

As part of RetS/GacS/GacA/RsmA regulatory pathway, the *retS* gene is important for phenotypic shifting from acute to chronic infections (Lapouge et al., 2008; Moscoso et al., 2011). The *retS* mutation repressed the production of virulence factors such as T3SS and swarming motility while it upregulated production of the T6SS (type 6 secretion system) and exopolysaccharides Pel/Psl required for biofilm formation (Moscoso et al., 2011; **Figure 4**). The T6SS is a puncturing device for delivery of proteins and toxins into the competing bacteria and the host cells and an important survival advantage for *P. aeruginosa*. It is also required for biofilm formation while being considered as a virulence factor (Chen et al., 2015). This transitional impact was shown to be mediated by high levels of cyclic di-GMP (Boehm et al., 2010; Paul et al., 2010; Moscoso et al., 2011).

Mutation of the *ampR* gene is another common mutational adaptation with a large impact on remodeling of physiological traits (**Figure 4**). The AmpR global regulator in *P. aeruginosa* regulates not only resistance to different classes of clinically relevant antibiotics, but also expression of hundreds of genes involved in diverse physiological processes such as virulence, QS systems and stress responses (Balasubramanian et al., 2015). It is understood that the *ampR* mutation induces adaptations leading to chronic infection including the downregulation of stress responses and virulence factors via downregulating QS systems, and boosting biofilm formation and alginate overproduction by causing elevation of cyclic di-GMP levels. Additionally, it induced AlgU activity, and resistance to fluoroquinolone through activation of MexT upregulating the MexEF-OprN efflux pump as well as increasing twitching motility and T6SS production (Balasubramanian et al., 2011, 2012, 2014, 2015; **Figure 4**).

Other adaptive mutations of CF isolates have been commonly reported in anti-mutator genes including *mutS*, *mutT*, *mutL*, *mutY*, *mutM*, and *uvrD* conferring a "hypermutability phenotype" with elevated mutation rates due to the lack of DNA repair mechanisms. This phenotype has been described as being caused by later mutational events as they are understood to occur after mutation of the *lasR* and *mucA* genes known as earlier mutations. However, other reports postulated that mutation of anti-mutator loci may increase the rate of other adaptive mutations (Oliver and Mena, 2010).

Hypermutators are very prevalent in CF isolates and they are shown to have correlation with higher antibiotic resistance particularly in the late stage of chronic infections. However, hypermutators also display other phenotypes such as mucoidity, lack of motility and LPS production (Oliver et al., 2000; Ciofu et al., 2010; Varga et al., 2015).

Other distinct phenotypes correlated with adaptation to CF airways are the small-colony variants (SCVs). They are associated with prolonged persistence and chronic infections in CF lungs and obstructive pulmonary diseases (Malone, 2015). They have been characterized as variants forming rugose small colonies on solid media (1–3 mm in diameter) with slow growing, autoaggregative and enhanced biofilm formation characteristics combined with enhanced surface attachment and hyperpiliation for twitching (Häussler et al., 1999, 2003; Kirisits et al., 2005). *In vitro* analyses showed that the SCVs display increased resistance to a wide range of antibiotics (Wei et al., 2011). Different studies have demonstrated that the presence of SCVs in the CF lung is associated with poorer lung function and clinical outcomes (Häussler et al., 1999, 2003; Schneider et al., 2008).

It has been understood that SCVs show high levels of cyclic di-GMP production aligned with increased production of Pel and Psl exopolysaccharides (Starkey et al., 2009). So far investigations regarding the molecular mechanisms underlying SCVs formation confirmed loss-of-function mutations in regulatory genes such as *wspF, yfiR* and *rsmA* and some other genes which alter regulatory networks in favor of enhanced cyclic di-GMP production (Irie et al., 2010; Malone et al., 2012; Blanka et al., 2015; Malone, 2015). On the other hand, an upregulating cyclic di-GMP synthesis pathway is a key determinant of exopolysaccharide production leading to highly developed biofilms. However, it still remains unclear how two distinct phenotypes i.e., cell within mucoid biofilm and SCV differ in regard to the cyclic di-GMP mediated signaling pathways.

SURVIVAL BY STRINGENT RESPONSE AND PERSISTER FORMATION

Stringent response to environmental stresses such as nutritional starvation and response to antibiotics and oxidative stresses share a similar outcome of adaptation i.e., all are leading to dormancy and persister formation. In both responses, bacteria slow down their metabolism through downregulating the expression of genes participating in the biosynthesis of proteins, ribosomes, cell wall, nucleic acid metabolism, and virulence factors. These dramatic metabolic alterations result in arresting cell growth and cell division in favor of bacterial survival (Eymann et al., 2002; Hesketh et al., 2007; Durfee et al., 2008).

Persisters are defined as subpopulations of cells, occurring at very low frequency, which stochastically emerge in the presence of stress. They show very slow growth enhancing survival under stress while viability of the majority of the population is severely impaired. Upon stress removal, persisters turn back to normal growth to propagate, which coincides with regained sensitivity to stress. Such persistence was suggested to be based on the heterogeneity of population by means of epigenetic mechanisms, not genetic mutations (Fasani and Savageau, 2015).

Various studies have provided evidences showing the link between stringent response and persistence, but mostly using *E. coli* as a model which can be informative for *P. aeruginosa* as it possesses homologous signaling pathways (Fung et al.,

2010; Maisonneuve et al., 2013; Amato et al., 2014; Ramisetty et al., 2016). There are only a few studies aiming to explain such responses, but they provided inconclusive explanations. Therefore, we summarized the general findings in order to propose the underlying molecular mechanisms in *P. aeruginosa*.

Molecular Mechanisms Underlying Stringent Responses and Persisters

Notably, increased levels of (p)ppGpp (collectively designated for guanosine pentaphosphate and guanosine tetraphosphate) molecules in the cells is a central triggering alarmone for both persistence and stringent response (Potrykus and Cashel, 2008; Wu et al., 2010; Amato et al., 2014). The cellular levels of (p)ppGpp are mediated by the activity of the (p)ppGpp-synthesizing and degrading enzymes such as RelA and SpoT in response to external stimuli (Bremer and Dennis, 2008).

In stringent response when *E. coli* encounters amino acid deprivation, the ribosome-associated RelA synthesizes ppGpp molecules to an upper level. In association with the transcriptional regulator DksA (global regulator of metabolism), ppGpp interacts with RNA polymerase and inhibits the transcription of ribosomal RNA promoters. This inhibitory impact is concomitant with activation and upregulation of pathways for amino acid biosynthesis and the transcription of stress response genes (Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012; Amato et al., 2014). Amato et al. (2013) found that stringent responses are linked to the emergence of persisters by involving ppGpp based regulatory events (Amato et al., 2013).

The persister state is typically based on the activity of genetically encoded toxin-antitoxin (TA) modules particularly in response to antibiotics, but proposed as being activated by the same acting elements i.e., RelA or SpoT and (p)ppGpp in *E. coli* (Maisonneuve et al., 2013). These TA system are widely distributed in genomes or plasmids of bacteria and archaea (Van Melderen, 2010). Basically, the toxin element is a stable protein while the cognate antitoxin element either is a protein or a small RNA molecule which are metabolically unstable under unfavorable conditions. A small RNA antitoxin directly inhibits the toxin translation by pairing with toxin mRNA or inactivate toxin by direct binding. A protein antitoxin on the other hand either degrades toxin mRNA or blocks the activity of cognate protein toxin via direct protein-protein interaction or protective interaction with toxin substrates. Impairment of antitoxin function under certain stresses such as antibiotics as well as nutritional stresses leads to the accumulation and activation of toxin proteins. Consequently, the toxin targets and interferes with key cellular processes such as DNA replication, and the synthesis of tRNA, membrane components, and ATP leading to the inhibition of cell growth and cell division to form a dormant or persister cell (Christensen-Dalsgaard et al., 2010; Wen et al., 2014). Presumably, bacteria switch off their active metabolism upon exposure to stress to evade starvation or antibiotic impact on their cellular targets. Christensen et al. demonstrated that *E. coli* encodes at least 10 TA loci and all of which could be induced by nutritional starvation and antibiotics (Christensen et al., 2001; Christensen-Dalsgaard et al., 2010).

So far, Maisonneuve et al. (2013) have proposed the best model presenting the hierarchical molecular mechanisms for *E. coli* persistence with the involvement of the key players including RelA/SpoT enzymes, (p)ppGpp signaling, Lon protease (ATP-dependent protease), inorganic polyphosphate (PolyP), and toxin-antitoxins systems (Maisonneuve et al., 2013; **Figure 5**). This model explains that (p)ppGpp synthesized by RelA/SpoT inhibits PolyP degradation, while PolyP accumulation stimulates Lon protease to degrade the antitoxin leading to the activation of toxin for arresting cellular processes and growth. Previously, it was shown in *E. coli* that in response to nutritional stress and antibiotics, transcriptional activation of TA loci depended on protease activity of Lon protease on antitoxin which itself repressed the TA promoter (Christensen-Dalsgaard et al., 2010). Furthermore, it was suggested that the complex of Lon protease with PolyP could promote ribosomal protein degradation for supplying required amino acids during starvation (Kuroda et al., 2001; **Figure 5**).

P. aeruginosa Stringent Response and Persisters

Required elements of stringent response and persistence including the ppGpp alarmone, SpoT, RelA, DksA, and the TA modules have been characterized in *P. aeruginosa* (**Figure 5**). Mutants deficient in *relA* and/or *spoT* genes showed increased sensitivity to heat shock, oxidative and osmotic stresses as well as antibiotics while becoming less virulent. Stringent response and RelA/SpoT activity for production of ppGpp were found to be crucial for regulation of virulence factor production (Erickson et al., 2004; Viducic et al., 2006; Boes et al., 2008; Nguyen et al., 2011; Vogt et al., 2011). Also, there is some experimental support that *P. aeruginosa* utilizes stringent response to protect cells from oxidative stresses generated by toxic reactive oxygen species (ROS) under aerobic conditions. The mutants deficient in *relA* and *spoT* genes were highly susceptible to multiple oxidants. This study showed that (p)ppGpp signaling is necessary for optimal expression of catalase and superoxide dismutase enzymes as major ROS scavengers, but it was assumed to be indirectly regulated through a complex regulatory network (Sampathkumar et al., 2016; **Figure 5**). Because, (p)ppGpp signaling is also required for full expression of other regulatory pathways controlling antioxidant response such as the stress response regulator RpoS as well as both Las and Rhl QS systems (van Delden et al., 2001; Kohanski et al., 2008; Schafhauser et al., 2014; Sampathkumar et al., 2016; **Figure 5**). The proposed *E. coli* model for the activation of the TA system via Lon protease has not been shown for *P. aeruginosa,* yet. However, different studies indicated that Lon protease activity was induced by aminoglycosides and *lon* mutants were highly susceptible to ciprofloxacin while *lon* was also required for biofilm formation, motility and virulence (Marr et al., 2007; Breidenstein et al., 2012). Molecular mechanism of these pathways have not been well characterized, but as proposed previously, interconnection of various regulatory and signaling pathways for appropriate responses leading to either as stringent/persistence, biofilm formation or virulence is anticipated (Kim et al., 1998).

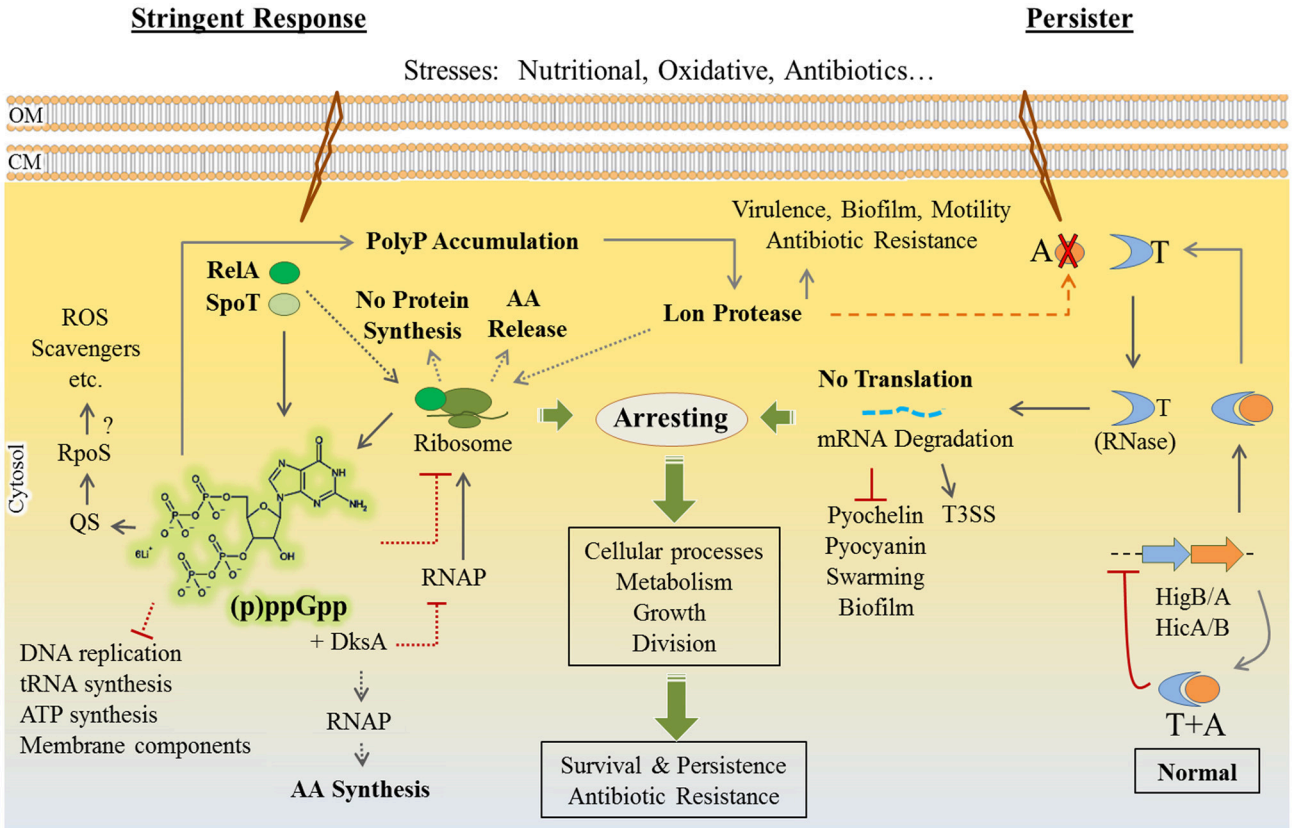


FIGURE 5 | *P. aeruginosa* stringent response and persister formation. Stringent response is triggered by particular stresses such as amino acid and fatty acid starvation, iron/phosphor depletion and oxidative stress [e.g., reactive oxygen species (ROS)]. The (p)ppGpp alarmone is a key determinant for stringent response and it is elevated by RelA/SpoT enzymes. Generally, (p)ppGpp elevation and the PolyP (inorganic polyphosphate) and Lon protease complex interfere with normal biological processes in favor of bacterial survival via arresting metabolism, cell growth and cell division (dashed gray pathways are best understood for the *E. coli* model, but not or partially characterized in *P. aeruginosa*). In *E.coli*, (p)ppGpp signaling is linked to toxin (T)-antitoxin (A) system via activation of the Lon protease leading to the formation of persisters displaying dormant and antibiotic resistance phenotypes (dashed orange line). Generally, the TA complex is stable under normal conditions suppressing toxin activity and further expression of cognate genes. Upon antitoxin degradation, toxin becomes active to hinder biological processes. In the case of *P. aeruginosa* HigB/A, HicA/B, the toxin components perform endoribonuclease (RNase) activity on mRNA molecules. In *P. aeruginosa,* the (p)ppGpp alarmone is linked to the production of ROS scavengers probably via QS or RpoS regulators and Lon activity is required for biofilm formation, motility, virulence and antibiotic resistance. Furthermore, the TA system downregulates biofilm formation and virulence factor production while T3SS (type 3 secretion system) can be found upregulated. Although, the (p)ppGpp signaling, Lon protease activity and TA modules (i.e., HigB/A, HicA/B, and likely more complexes) are present in *P. aeruginosa,* their link to resistance to antibiotics and other stresses is poorly understood. AA, amino acids; QS, quorum sensing; RNAP, RNA polymerase. CM, cytoplasmic membrane; OM, outer membrane.

To date five types (I-V) of TA systems have been described in bacteria based on the nature and mode of action of antitoxin (Wang et al., 2012). So far, HigB/HigA and HicA/HicB TA modules encoded by genomic loci have been experimentally demonstrated in *P. aeruginosa*, while other TA systems such as the *relBE* and *parDE* loci were predicted but they have not been characterized, yet (Pandey and Gerdes, 2005; Fernández-García et al., 2016; **Figure 5**). The HigB/HigA and HicA/HicB TA modules have been also widely reported for other bacteria (Pandey and Gerdes, 2005; Li G. et al., 2016; Wood and Wood, 2016).

These TA modules belong to the type II TA system where both toxin (i.e., HigB/HicA) and antitoxin (i.e., HigA/HicB) are proteins directly interacting with each other retaining the toxin inactivated, such as inhibiting the RNase activity of HigB or HicA (Christensen et al., 2001; Rocker and Meinhart, 2016; **Figure 5**). The HigB/HigA TA module was found to influence *P. aeruginosa* pathogenicity where toxin HigB was shown to reduce the production of the virulence factors pyochelin, pyocyanin, swarming, and impaired biofilm formation representing a novel function for a TA systems (Wood and Wood, 2016; **Figure 5**). Another study showed that the HigB/HigA TA module was necessary for the ciprofloxacin induced persister formation by *P. aeruginosa*. Concurrently, HigB overproduction upregulated the expression of T3SS genes leading to the accumulation of T3SS proteins in persisters as well as increasing bacterial cytotoxicity against host immune cells (Li M.et al., 2016; **Figure 5**).

Furthermore, these TA systems have been shown to be highly prevalent in the clinical strains (Pandey and Gerdes, 2005; Williams et al., 2011; Li G. et al., 2016). It is believed that

persisters are one of the main reasons for recurring and chronic infections where persisters withstand antibiotic treatments and spawn new infecting population upon removal of antibiotic treatment (Lewis, 2007; Wang and Wood, 2011). They are abundant in *P. aeruginosa* biofilms which is the hallmark of long-term infections particularly in CF patients (Lewis, 2008; Mulcahy et al., 2010).

P. aeruginosa RESISTANCE TO FOREIGN DNA

Infection of bacteria with viruses or bacteriophages is a natural phenomenon which can lead to bacterial lysis. Bacteria harness various mechanisms to destroy such foreign DNAs leading to resistance. The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated proteins) systems form the only adaptive immune system in prokaryotic cells which also mediates *P. aeruginosa* survival during viral invasions (Cady et al., 2012; Bondy-Denomy and Davidson, 2014). A CRISPR region is an array of multiple repeated sequences on the bacterial genome or a plasmid ranging from 21 to 48 bp in length and separated by 26 to 72 bp hypervariable spacers (Bhaya et al., 2011; Cady et al., 2012). The *cas* locus encoding Cas proteins is located in the vicinity of the CRISPR region (Bhaya et al., 2011). In principle, the molecular mechanism is based on acquisition and integration of small fragments of foreign DNAs such as derived from viruses into the spacer regions between two adjacent repeats within the CRISPR locus mediated by Cas proteins with nuclease activity. Subsequently, the CRISPR region is transcribed resulting in pre-CRISPR RNA (pre-crRNA) which undergoes hydrolysis by endoribonucleases forming small CRISPR RNAs (crRNAs). The mature crRNAs in association with a multiprotein complex known as CASCADE (CRISPR-associated complex for antiviral defense) recognizes invasive DNAs upon complementarity which results in the initiation of the cleavage of the crRNA–foreign DNA hybridization complex, mediating survival of bacteria after viral infections while protecting themselves from lysis (Brouns et al., 2008; Mojica et al., 2009; Deveau et al., 2010; Garneau et al., 2010; Bhaya et al., 2011). The CRISPR-Cas systems have been classified into three major types (I, II and III) and at least 11 subtypes (IA-F, IIA-C and IIIA-B) encoding distinct crRNA-guided surveillance complexes (Makarova et al., 2011).

A study showed that 36% of tested *P. aeruginosa* clinical isolates harbored CRISPR-Cas systems developing adaptive immunity against various mobile genetic elements such as temperate phages, prophages, pathogenicity island transposons which were integrated into the genome (Cady et al., 2011). Different studies demonstrated that the types I-F and I-E CRISPR-Cas systems are naturally active in *P. aeruginosa* isolates (Cady et al., 2012; Pawluk et al., 2014). A recent phylogenetic study revealed the existence of the type I-C CRISPR-Cas system in some isolates of *P. aeruginosa* (van Belkum et al., 2015).

On the other hand, the activity of CRISPR/Cas system can be inhibited by anti-CRISPR/Cas genes harbored by phages infecting *P. aeruginosa* which counteract the type I-F and I-E systems (Bondy-Denomy et al., 2015; Maxwell, 2016). Also it has been shown that phages producing anti-CRISPR activity are closely related to each other and with high sequence similarity to bacteriophage DMS3 (Bondy-Denomy et al., 2013). Bacteriophage DMS3 was isolated from clinical isolates of *P. aeruginosa* and it was shown to inhibit biofilm formation and swarming motility, and *P. aeruginosa* cannot develop immunity against it due to the lack of complementarity between crRNA and protospacers of DMS3 genome (Budzik et al., 2004; Zegans et al., 2009). Furthermore, the CRISPR-Cas systems show a strong correlation with antibiotic resistance/susceptibility (van Belkum et al., 2015). Additionally, the same study showed that the CRISPR-Cas systems play an important role in shaping the accessory genomes of globally distributed *P. aeruginosa* strains. Accessory genome is referred to highly variable regions of the genome versus a relatively invariable core genome. *P. aeruginosa* accessory genome varies from strain to strain, ranging from 6.9 to 18.0% of the total genome, and is mainly comprised of integrative and conjugative elements, replacement islands, prophages and phage-like elements, transposons, insertion sequences and integrons (Kung et al., 2010; Ozer et al., 2014). According to this finding the CRISPR typing with regard to the frequency of spacer integration and deletion between related strains can potentially be used for identifying the lineage of strains especially within outbreaks (van Belkum et al., 2015).

Overall, understanding of the CRISPR-Cas and anti-CRISPR-Cas systems is gradually becoming important in the context of pathogenesis and strain lineage identification. These links were highlighted by the discovery of the interaction of bacteriophage DMS3 and the type I-F CRISPR, and its impact on biofilms (Zegans et al., 2009; Palmer and Whiteley, 2011) as well as the role of different CRISPR/Cas systems on virulence and antibiotic resistance (Louwen et al., 2014). Overall, these findings suggest a more diverse function of CRISPR/Cas systems within the context of pathogenesis, requiring further in depth studies to elucidate the underlying molecular mechanisms.

CONCLUSIONS AND PERSPECTIVES

For many years, *P. aeruginosa* has been a model organism and received much attention from scientific community to study the bacterial lifestyle and pathogenesis. It always has been of particular importance due to causing persistent infections in CF and immunocompromised patients. Nowadays, this ubiquitous bacterial pathogen is accepted worldwide as a public health risk due to its increasing prevalence in healthcare acquired infections combined with its ability to develop resistances to multiple classes of antibiotics. Over the past decade, extensive research studies have focused on these growing concerns aiming at deciphering the nature of *P. aeruginosa* capability and underlying molecular mechanisms applying different modes of persistence and antibiotic resistance.

In this review, we summarized several of the well characterized molecular mechanisms which enable *P. aeruginosa* to survive various hostile conditions such as during pathogenesis and antibiotic treatment. These mechanisms form multiple layers of physiological adaptations correlating with social behavior and lifestyle of bacteria while responding environmental stimuli. Such

extraordinary adaptive capability relies on extensive numbers of regulatory or controlling factors within integrated and complex signal processing pathways. These enable bacteria perceive and process environmental cues in order orchestrate physiological changes to promote adaptation to unfavorable conditions. Many of these regulatory pathways, their cognate player, their signals and how they are integrated with global regulatory networks still remain poorly understood.

Furthermore, we highlighted key molecular pathways driving *P. aeruginosa* survival and persistence at different stages of pathogenesis such as QS elements for virulence traits, cyclic di-GMP signaling in biofilm formation and development of chronic traits, (p)ppGpp signaling/TA system in persister formation and various strategic adaptations for developing resistance to divers classes of antibiotics.

Recent technological advances in genomic characterization of pathogens have provided invaluable information about the dynamics of *P. aeruginosa* populations and their heterogeneity at different stages of pathogenesis. These results which were explained under "adaptive radiation" term emphasized that this species shows a stunning capability to become resilient during pathogenesis to withstand antibacterial treatment.

Available information indicated that sole therapy which only relies on bacteriostatic/bactericidal compounds can readily be defeated by bacterial resiliency and a management program is still required to combat infections. This program should be able to predict and evaluate physiological adaptations at each stage of infection for exerting appropriate treatments which could interfere with adaptation rather than increasing the chance of bacterial survival. A good example is improper and frequent application of antibiotics which must be avoided. Instead a comprehensive hygiene program must be applied in healthcare settings and among personnel to stop the spread of nosocomial infections specifically caused by multidrug resistance strains. Also, further research on identification of new drugs and developing new alternative prevention and treatment strategies for interfering with key regulatory pathways is needed.

At the end we suggest all efforts should consider international coordinated multidisciplinary programs with results of laboratory outputs being deposited in centralized accessible databases to expedite advances in control of infections and its implementation into clinical settings. The steadily growing concern of emerging antibiotic resistance strains in the world, would justify the set-up of such databases which then allow developing world-wide guidelines for monitoring and recording antibiotic resistance cases around the world. This should provide healthcare experts with appropriate guidelines for well managing bacterial infections and preventing the rate and spread of resistance strains.

AUTHOR CONTRIBUTIONS

MM and BR conceived and wrote the majority of the manuscript. SG contributed biofilm and alginate related aspects to the manuscript.

FUNDING

The work was funded via the Massey University Research fund.

ACKNOWLEDGMENTS

The authors are grateful to all past and present members of the BR Research group at the Institute of Fundamental Sciences (Massey University) for their contributions to the *Pseudomonas aeruginosa* research.

REFERENCES

Aendekerk, S., Diggle, S. P., Song, Z., Høiby, N., Cornelis, P., Williams, P., et al. (2005). The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinolone-dependent cell-to-cell communication. *Microbiology* 151(Pt 4), 1113–1125. doi: 10.1099/mic.0.27631-0

Alcalde-Rico, M., Hernando-Amado, S., Blanco, P., and Martínez, J. L. (2016). Multidrug efflux pumps at the crossroad between antibiotic resistance and bacterial virulence. *Front. Microbiol.* 7:1483. doi: 10.3389/fmicb.2016.01483

Alhede, M., Bjarnsholt, T., Jensen, P. Ø., Phipps, R. K., Moser, C., Christophersen, L., et al. (2009). *Pseudomonas aeruginosa* recognizes and responds aggressively to the presence of polymorphonuclear leukocytes. *Microbiology* 155(Pt 11), 3500–3508. doi: 10.1099/mic.0.031443-0

Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., et al. (2006). A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol. Microbiol.* 59, 1114–1128. doi: 10.1111/j.1365-2958.2005.05008.x

Alvarez-Ortega, C., and Harwood, C. S. (2007). Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Mol. Microbiol.* 65, 153–165. doi: 10.1111/j.1365-2958.2007.05772.x

Amato, S. M., Fazen, C. H., Henry, T. C., Mok, W. W., Orman, M. A., Sandvik, E. L., et al. (2014). The role of metabolism in bacterial persistence. *Front. Microbiol.* 5:70. doi: 10.3389/fmicb.2014.00070

Amato, S. M., Orman, M. A., and Brynildsen, M. P. (2013). Metabolic control of persister formation in *Escherichia coli*. *Mol. Cell* 50, 475–487. doi: 10.1016/j.molcel.2013.04.002

Amiel, E., Lovewell, R. R., O'Toole, G. A., Hogan, D. A., and Berwin, B. (2010). *Pseudomonas aeruginosa* evasion of phagocytosis is mediated by loss of swimming motility and is independent of flagellum expression. *Infect. Immun.* 78, 2937–2945. doi: 10.1128/IAI.00144-10

Avrain, L., Mertens, P., and Van Bambeke, F. (2013). RND efflux pumps in *P. aeruginosa*: an underestimated resistance mechanism. *Antibiot. Susceptibility* 26321, 26–28. Available online at: http://www.uclouvain.be/cps/ucl/doc/ir-ldri/images/Avrain-2013-1.pdf

Ayers, M., Sampaleanu, L. M., Tammam, S., Koo, J., Harvey, H., Howell, P. L., et al. (2009). PilM/N/O/P proteins form an inner membrane complex that affects the stability of the *pseudomonas aeruginosa* type iv pilus secretin. *J. Mol. Biol.* 394, 128–142. doi: 10.1016/j.jmb.2009.09.034

Bains, M., Fernández, L., and Hancock, R. E. (2012). Phosphate starvation promotes swarming motility and cytotoxicity of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 78, 6762–6768. doi: 10.1128/AEM.01015-12

Bajolet-Laudinat, O., Girod-de Bentzmann, S., Tournier, J. M., Madoulet, C., Plotkowski, M. C., Chippaux, C., et al. (1994). Cytotoxicity of *Pseudomonas aeruginosa* internal lectin PA-I to respiratory epithelial cells in primary culture. *Infect. Immun.* 62, 4481–4487.

Balasubramanian, D., Kong, K. F., Jayawardena, S. R., Leal, S. M., Sautter, R. T., and Mathee, K. (2011). Co-regulation of β-lactam resistance, alginate production and quorum sensing in *Pseudomonas aeruginosa*. *J. Med. Microbiol.* 60(Pt 2), 147–156. doi: 10.1099/jmm.0.021600-0

Balasubramanian, D., Kumari, H., Jaric, M., Fernandez, M., Turner, K. H., Dove, S. L., et al. (2014). Deep sequencing analyses expands the *Pseudomonas aeruginosa* AmpR regulon to include small RNA-mediated regulation of iron acquisition, heat shock and oxidative stress response. *Nucleic Acids Res.* 42, 979–998. doi: 10.1093/nar/gkt942

Balasubramanian, D., Kumari, H., and Mathee, K. (2015). *Pseudomonas aeruginosa* AmpR: an acute-chronic switch regulator. *Pathog. Dis.* 73, 1–14. doi: 10.1111/2049-632X.12208

Balasubramanian, D., Schneper, L., Merighi, M., Smith, R., Narasimhan, G., Lory, S., et al. (2012). The regulatory repertoire of *Pseudomonas aeruginosa* AmpC ss-lactamase regulator AmpR includes virulence genes. *PLoS ONE* 7:e34067. doi: 10.1371/journal.pone.0034067

Banerjee, G., and Ray, A. K. (2016). The talking language in some major Gram-negative bacteria. *Arch. Microbiol.* 198, 489–499. doi: 10.1007/s00203-016-1220-x

Baraquet, C., Murakami, K., Parsek, M. R., and Harwood, C. S. (2012). The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the pel operon promoter in response to c-di-GMP. *Nucleic Acids Res.* 40, 7207–7218. doi: 10.1093/nar/gks384

Barclay, M. L., Begg, E. J., and Chambers, S. T. (1992). Adaptive resistance following single doses of gentamicin in a dynamic *in vitro* model. *Antimicrob. Agents Chemother.* 36, 1951–1957. doi: 10.1128/AAC.36.9.1951

Barken, K. B., Pamp, S. J., Yang, L., Gjermansen, M., Bertrand, J. J., Klausen, M., et al. (2008). Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ. Microbiol.* 10, 2331–2343. doi: 10.1111/j.1462-2920.2008.01658.x

Barker, A. P., Vasil, A. I., Filloux, A., Ball, G., Wilderman, P. J., and Vasil, M. L. (2004). A novel extracellular phospholipase C of *Pseudomonas aeruginosa* is required for phospholipid chemotaxis. *Mol. Microbiol.* 53, 1089–1098. doi: 10.1111/j.1365-2958.2004.04189.x

Barr, H. L., Halliday, N., Cámara, M., Barrett, D. A., Williams, P., Forrester, D. L., et al. (2015). *Pseudomonas aeruginosa* quorum sensing molecules correlate with clinical status in cystic fibrosis. *Eur. Respir. J.* 46, 1046–1054. doi: 10.1183/09031936.00225214

Barrow, K., and Kwon, D. H. (2009). Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 5150–5154. doi: 10.1128/AAC.00893-09

Baumgart, A. M., Molinari, M. A., and Silveira, A. C. (2010). Prevalence of carbapenem resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in high complexity hospital. *Braz. J. Infect. Dis.* 14, 433–436. doi: 10.1590/S1413-86702010000500002

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br. J. Pharmacol.* 153(Suppl. 1), S347–357. doi: 10.1038/sj.bjp.0707607

Berrazeg, M., Jeannot, K., Ntsogo Enguene, V. Y., Broutin, I., Loeffert, S., Fournier, D., et al. (2015). Mutations in β-lactamase AmpC increase resistance of *Pseudomonas aeruginosa* isolates to antipseudomonal cephalosporins. *Antimicrob. Agents Chemother.* 59, 6248–6255. doi: 10.1128/AAC.00825-15

Bhaya, D., Davison, M., and Barrangou, R. (2011). CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* 45, 273–297. doi: 10.1146/annurev-genet-110410-132430

Billings, N., Millan, M., Caldara, M., Rusconi, R., Tarasova, Y., Stocker, R., et al. (2013). The extracellular matrix component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog.* 9:e1003526. doi: 10.1371/journal.ppat.1003526

Bjarnsholt, T., Jensen, P. Ø., Jakobsen, T. H., Phipps, R., Nielsen, A. K., Rybtke, M. T., et al. (2010). Quorum sensing and virulence of *Pseudomonas aeruginosa* during lung infection of cystic fibrosis patients. *PLoS ONE* 5:e10115. doi: 10.1371/journal.pone.0010115

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., and Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* 13, 42–51. doi: 10.1038/nrmicro3380

Blanka, A., Düvel, J., Dotsch, A., Klinkert, B., Abraham, W. R., Kaever, V., et al. (2015). Constitutive production of c-di-GMP is associated with mutations in a variant of *Pseudomonas aeruginosa* with altered membrane composition. *Sci. Signal.* 8:ra36. doi: 10.1126/scisignal.2005943

Blumer, C., and Haas, D. (2000). Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Arch. Microbiol.* 173, 170–177. doi: 10.1007/s002039900127

Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C. A., Ackermann, M., et al. (2010). Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* 141, 107–116. doi: 10.1016/j.cell.2010.01.018

Boes, N., Schreiber, K., and Schobert, M. (2008). SpoT-triggered stringent response controls usp gene expression in *Pseudomonas aeruginosa*. *J. Bacteriol.* 190, 7189–7199. doi: 10.1128/JB.00600-08

Boles, B. R., and Singh, P. K. (2008). Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12503–12508. doi: 10.1073/pnas.0801499105

Boles, B. R., Thoendel, M., and Singh, P. K. (2005). Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol. Microbiol.* 57, 1210–1223. doi: 10.1111/j.1365-2958.2005.04743.x

Bondy-Denomy, J., and Davidson, A. R. (2014). To acquire or resist: the complex biological effects of CRISPR-Cas systems. *Trends Microbiol.* 22, 218–225. doi: 10.1016/j.tim.2014.01.007

Bondy-Denomy, J., Garcia, B., Strum, S., Du, M. J., Rollins, M. F., Hidalgo-Reyes, Y., et al. (2015). Multiple mechanisms for CRISPR-Cas inhibition by anti-CRISPR proteins. *Nature* 526, 136–139. doi: 10.1038/nature15254

Bondy-Denomy, J., Pawluk, A., Maxwell, K. L., and Davidson, A. R. (2013). Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature* 493, 429–432. doi: 10.1038/nature11723

Breidenstein, E. B. M., Janot, L., Strehmel, J., Fernandez, L., Taylor, P. K., Kukavica-Ibrulj, I., et al. (2012). The Lon protease is essential for full virulence in *Pseudomonas aeruginosa*. *PLoS ONE* 7:e49123. doi: 10.1371/journal.pone.0049123

Bremer, H., and Dennis, P. P. (2008). Modulation of chemical composition and other parameters of the cell at different exponential growth rates. *EcoSal Plus* 3, 1–49. doi: 10.1128/ecosal.5.2.3

Britigan, B. E., Roeder, T. L., Rasmussen, G. T., Shasby, D. M., McCormick, M. L., and Cox, C. D. (1992). Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to endothelial cells - Implications for *Pseudomonas* associated tissue injury. *J. Clin. Invest.* 90, 2187–2196. doi: 10.1172/JCI116104

Brouns, S. J., Jore, M. M., Lundgren, M., Westra, E. R., Slijkhuis, R. J., Snijders, A. P., et al. (2008). Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321, 960–964. doi: 10.1126/science.1159689

Budzik, J. M., Rosche, W. A., Rietsch, A., and O'Toole, G. A. (2004). Isolation and characterization of a generalized transducing phage for *Pseudomonas aeruginosa* strains PAO1 and PA14. *J. Bacteriol.* 186, 3270–3273. doi: 10.1128/JB.186.10.3270-3273.2004

Burrows, L. L. (2012). *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annu. Rev. Microbiol.* 66, 493–520. doi: 10.1146/annurev-micro-092611-150055

Byrd, M. S., Sadovskaya, I., Vinogradov, E., Lu, H., Sprinkle, A. B., Richardson, S. H., et al. (2009). Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Mol. Microbiol.* 73, 622–638. doi: 10.1111/j.1365-2958.2009.06795.x

Cady, K. C., Bondy-Denomy, J., Heussler, G. E., Davidson, A. R., and O'Toole, G. A. (2012). The CRISPR/Cas adaptive immune system of *Pseudomonas aeruginosa* mediates resistance to naturally occurring and engineered phages. *J. Bacteriol.* 194, 5728–5738. doi: 10.1128/JB.01184-12

Cady, K. C., White, A. S., Hammond, J. H., Abendroth, M. D., Karthikeyan, R. S., Lalitha, P., et al. (2011). Prevalence, conservation and functional analysis of Yersinia and Escherichia CRISPR regions in clinical *Pseudomonas aeruginosa* isolates. *Microbiology* 157(Pt 2), 430–437. doi: 10.1099/mic.0.045732-0

Cai, Z., Liu, Y., Chen, Y., Yam, J. K., Chew, S. C., Chua, S. L., et al. (2015). RpoN regulates virulence factors of *Pseudomonas aeruginosa* via modulating the PqsR quorum sensing regulator. *Int. J. Mol. Sci.* 16, 28311–28319. doi: 10.3390/ijms161226103

Cardo, D., Horan, T., Andrus, M., Dembinski, M., Edwards, J., Peavy, G., et al. (2004). National nosocomial infections surveillance (NNIS) system report, data summary from January 1992 through June 2004, issued October 2004. *Am. J. Infect. Control* 32, 470–485. doi: 10.1016/j.ajic.2004.10.001

Castric, P. (1994). Influence of oxygen on the *Pseudomonas aeruginosa* hydrogen cyanide synthase. *Curr. Microbiol.* 29, 19–21. doi: 10.1007/BF01570186

Castric, P. A. (1975). Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* 21, 613–618. doi: 10.1139/m75-088

Castric, P. A. (1983). Hydrogen cyanide production by *Pseudomonas aeruginosa* at reduced oxygen levels. *Can. J. Microbiol.* 29, 1344–1349. doi: 10.1139/m83-209

Chemani, C., Imberty, A., de Bentzmann, S., Pierre, M., Wimmerová, M., Guery, B. P., et al. (2009). Role of LecA and LecB lectins in *Pseudomonas aeruginosa*-induced lung injury and effect of carbohydrate ligands. *Infect. Immun.* 77, 2065–2075. doi: 10.1128/IAI.01204-08

Chen, L., Zou, Y., She, P., and Wu, Y. (2015). Composition, function, and regulation of T6SS in *Pseudomonas aeruginosa*. *Microbiol. Res.* 172, 19–25. doi: 10.1016/j.micres.2015.01.004

Choi, Y., Park, H. Y., Park, S. J., Park, S. J., Kim, S. K., Ha, C., et al. (2011). Growth phase-differential quorum sensing regulation of anthranilate metabolism in *Pseudomonas aeruginosa*. *Mol. Cells* 32, 57–65. doi: 10.1007/s10059-011-2322-6

Christensen, S. K., Mikkelsen, M., Pedersen, K., and Gerdes, K. (2001). RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14328–14333. doi: 10.1073/pnas.251327898

Christensen-Dalsgaard, M., Jørgensen, M. G., and Gerdes, K. (2010). Three new RelE-homologous mRNA interferases of *Escherichia coli* differentially induced by environmental stresses. *Mol. Microbiol.* 75, 333–348. doi: 10.1111/j.1365-2958.2009.06969.x

Chrzanowski, L., Lawniczak, L., and Czaczyk, K. (2012). Why do microorganisms produce rhamnolipids? *World J. Microb. Biot.* 28, 401–419. doi: 10.1007/s11274-011-0854-8

Chugani, S., and Greenberg, E. P. (2007). The influence of human respiratory epithelia on *Pseudomonas aeruginosa* gene expression. *Microb. Pathog.* 42, 29–35. doi: 10.1016/j.micpath.2006.10.004

Ciofu, O., Mandsberg, L. F., Bjarnsholt, T., Wassermann, T., and Hoiby, N. (2010). Genetic adaptation of *Pseudomonas aeruginosa* during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogeneous genetic backgrounds emerge in *mucA* and/or *lasR* mutants. *Microbiology* 156, 1108–1119. doi: 10.1099/mic.0.033993-0

Colvin, K. M., Gordon, V. D., Murakami, K., Borlee, B. R., Wozniak, D. J., Wong, G. C. L., et al. (2011). The Pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog.* 7:e1001264 . doi: 10.1371/journal.ppat.1001264

Colvin, K. M., Irie, Y., Tart, C. S., Urbano, R., Whitney, J. C., Ryder, C., et al. (2012). The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environ. Microbiol.* 14, 1913–1928. doi: 10.1111/j.1462-2920.2011.02657.x

Daddaoua, A., Fillet, S., Fernández, M., Udaondo, Z., Krell, T., and Ramos, J. L. (2012). Genes for carbon metabolism and the ToxA virulence factor in *Pseudomonas aeruginosa* are regulated through molecular interactions of PtxR and PtxS. *PLoS ONE* 7:e39390. doi: 10.1371/journal.pone.0039390

Dalebroux, Z. D., and Swanson, M. S. (2012). ppGpp: magic beyond RNA polymerase. *Nat. Rev. Microbiol.* 10, 203–212. doi: 10.1038/nrmicro2720

D'Argenio, D. A., Wu, M., Hoffman, L. R., Kulasekara, H. D., Déziel, E., Smith, E. E., et al. (2007). Growth phenotypes of *Pseudomonas aeruginosa lasR* mutants adapted to the airways of cystic fibrosis patients. *Mol. Microbiol.* 64, 512–533. doi: 10.1111/j.1365-2958.2007.05678.x

da Silva, L. V. R. F., Ferreira, F. D., Reis, F. J. C., de Britto, M. C. A., Levy, C. E., Clark, O., et al. (2013). *Pseudomonas aeruginosa* infection in patients with cystic fibrosis: scientific evidence regarding clinical impact, diagnosis, and treatment. *J. Bras. Pneumol.* 39, 495–512. doi: 10.1590/S1806-37132013000400015

Das, T., Kutty, S. K., Kumar, N., and Manefield, M. (2013). Pyocyanin facilitates extracellular DNA binding to *Pseudomonas aeruginosa* influencing cell surface properties and aggregation. *PLoS ONE* 8:e58299. doi: 10.1371/journal.pone.0058299

Das, T., Kutty, S. K., Tavallaie, R., Ibugo, A. I., Panchompoo, J., Sehar, S., et al. (2015). Phenazine virulence factor binding to extracellular DNA is important for *Pseudomonas aeruginosa* biofilm formation. *Sci. Rep.* 5:8398. doi: 10.1038/srep08398

Davey, M. E., Caiazza, N. C., and O'Toole, G. A. (2003). Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 185, 1027–1036. doi: 10.1128/JB.185.3.1027-1036.2003

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74, 417–433. doi: 10.1128/MMBR.00016-10

Davies, J. E. (1997). Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found. Symp.* 207, 15–27; discussion 27–35.

Deveau, H., Garneau, J. E., and Moineau, S. (2010). CRISPR/Cas system and its role in phage-bacteria interactions. *Annu. Rev. Microbiol.* 64, 475–493. doi: 10.1146/annurev.micro.112408.134123

Diaz Caballero, J., Clark, S. T., Coburn, B., Zhang, Y., Wang, P. W., Donaldson, S. L., et al. (2015). Selective sweeps and parallel pathoadaptation drive *Pseudomonas aeruginosa* evolution in the cystic fibrosis lung. *MBio* 6, e00981–e00915. doi: 10.1128/mBio.00981-15

Diggle, S. P., Stacey, R. E., Dodd, C., Cámara, M., Williams, P., and Winzer, K. (2006). The galactophilic lectin, LecA, contributes to biofilm development in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 8, 1095–1104. doi: 10.1111/j.1462-2920.2006.001001.x

Diggle, S. P., Winzer, K., Lazdunski, A., Williams, P., and Cámara, M. (2002). Advancing the quorum in *Pseudomonas aeruginosa*: mvat and the regulation of N-acylhomoserine lactone production and virulence gene expression. *J. Bacteriol.* 184, 2576–2586. doi: 10.1128/JB.184.10.2576-2586.2002

Döring, G., Conway, S. P., Heijerman, H. G., Hodson, M. E., Høiby, N., Smyth, A., et al. (2000). Antibiotic therapy against *Pseudomonas aeruginosa* in cystic fibrosis: a European consensus. *Eur. Respir. J.* 16, 749–767. doi: 10.1034/j.1399-3003.2000.16d30.x

Driffield, K., Miller, K., Bostock, J. M., O'Neill, A. J., and Chopra, I. (2008). Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J. Antimicrob. Chemother.* 61, 1053–1056. doi: 10.1093/jac/dkn044

Durfee, T., Hansen, A. M., Zhi, H., Blattner, F. R., and Jin, D. J. (2008). Transcription profiling of the stringent response in *Escherichia coli*. *J. Bacteriol.* 190, 1084–1096. doi: 10.1128/JB.01092-07

Dusane, D. H., Zinjarde, S. S., Venugopalan, V. P., McLean, R. J., Weber, M. M., and Rahman, P. K. (2010). Quorum sensing: implications on rhamnolipid biosurfactant production. *Biotechnol. Genet. Eng. Rev.* 27, 159–184. doi: 10.1080/02648725.2010.10648149

El Amin, N., Giske, C. G., Jalal, S., Keijser, B., Kronvall, G., and Wretlind, B. (2005). Carbapenem resistance mechanisms in *Pseudomonas aeruginosa:* alterations of porin OprD and efflux proteins do not fully explain resistance patterns observed in clinical isolates. *APMIS* 113, 187–196. doi: 10.1111/j.1600-0463.2005.apm1130306.x

Epp, S. F., Köhler, T., Plésiat, P., Michéa-Hamzehpour, M., Frey, J., and Pechère, J. C. (2001). C-terminal region of *Pseudomonas aeruginosa* outer membrane porin OprD modulates susceptibility to meropenem. *Antimicrob. Agents Chemother.* 45, 1780–1787. doi: 10.1128/AAC.45.6.1780-1787.2001

Erickson, D. L., Lines, J. L., Pesci, E. C., Venturi, V., and Storey, D. G. (2004). *Pseudomonas aeruginosa* relA contributes to virulence in *Drosophila melanogaster*. *Infect. Immun.* 72, 5638–5645. doi: 10.1128/IAI.72.10.5638-5645.2004

Eurosurveillance Editorial Team (2013). CDC publishes report on antibiotic resistance threats in the United States for the first time. *Eurosurveillance* 18:5. Available online at: http://www.ecdc.europa.eu/en/activities/sciadvice/_layouts/forms/Review_DispForm.aspx?List=a3216f4c-f040-4f51-9f77-a96046dbfd72&ID=752&RootFolder=%2Fen%2Factivities%2Fsciadvice%2FLists%2FECDC%20Reviews&Web=0be238ef-4498-4962-8826-2e082a1f1639

Eymann, C., Homuth, G., Scharf, C., and Hecker, M. (2002). *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *J. Bacteriol.* 184, 2500–2520. doi: 10.1128/JB.184.9.2500-2520.2002

Falagas, M. E., and Kasiakou, S. K. (2005). Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin. Infect. Dis.* 40, 1333–1341. doi: 10.1086/429323

Fasani, R. A., and Savageau, M. A. (2015). Unrelated toxin-antitoxin systems cooperate to induce persistence. *J. R. Soc. Interface* 12:20150130. doi: 10.1098/rsif.2015.0130

Feng, L., Xiang, Q., Ai, Q., Wang, Z., Zhang, Y., and Lu, Q. (2016). Effects of quorum sensing systems on regulatory T cells in catheter-related *Pseudomonas aeruginosa* biofilm infection rat models. *Mediators Inflamm.* 2016:4012912. doi: 10.1155/2016/4012912

Fernández, L., Breidenstein, E. B., and Hancock, R. E. (2011). Creeping baselines and adaptive resistance to antibiotics. *Drug Resist. Updat.* 14, 1–21. doi: 10.1016/j.drup.2011.01.001

Fernández, L., Gooderham, W. J., Bains, M., McPhee, J. B., Wiegand, I., and Hancock, R. E. (2010). Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob. Agents Chemother.* 54, 3372–3382. doi: 10.1128/AAC.00242-10

Fernández, L., Jenssen, H., Bains, M., Wiegand, I., Gooderham, W. J., and Hancock, R. E. (2012). The two-component system CprRS senses cationic peptides and triggers adaptive resistance in *Pseudomonas aeruginosa* independently of ParRS. *Antimicrob. Agents Chemother.* 56, 6212–6222. doi: 10.1128/AAC.01530-12

Fernández-García, L., Blasco, L., Lopez, M., Bou, G., García-Contreras, R., Wood, T., et al. (2016). Toxin-antitoxin systems in clinical pathogens. *Toxins (Basel).* 8:227. doi: 10.3390/toxins8070227

Flemming, H. C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi: 10.1038/nrmicro2415

Flume, P. A., and Van Devanter, D. R. (2012). State of progress in treating cystic fibrosis respiratory disease. *BMC Med.* 10:88. doi: 10.1186/1741-7015-10-88

Folkesson, A., Jelsbak, L., Yang, L., Johansen, H. K., Ciofu, O., Høiby, N., et al. (2012). Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. *Nat. Rev. Microbiol.* 10, 841–851. doi: 10.1038/nrmicro2907

Franklin, M. J., Nivens, D. E., Weadge, J. T., and Howell, P. L. (2011). Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Front. Microbiol.* 2:167. doi: 10.3389/fmicb.2011.00167

Fraud, S., Campigotto, A. J., Chen, Z., and Poole, K. (2008). MexCD-OprJ multidrug efflux system of *Pseudomonas aeruginosa*: involvement in chlorhexidine resistance and induction by membrane-damaging agents dependent upon the AlgU stress response sigma factor. *Antimicrob. Agents Chemother.* 52, 4478–4482. doi: 10.1128/AAC.01072-08

Frimmersdorf, E., Horatzek, S., Pelnikevich, A., Wiehlmann, L., and Schomburg, D. (2010). How *Pseudomonas aeruginosa* adapts to various environments: a metabolomic approach. *Environ. Microbiol.* 12, 1734–1747. doi: 10.1111/j.1462-2920.2010.02253.x

Frisk, A., Schurr, J. R., Wang, G., Bertucci, D. C., Marrero, L., Hwang, S. H., et al. (2004). Transcriptome analysis of *Pseudomonas aeruginosa* after interaction with human airway epithelial cells. *Infect. Immun.* 72, 5433–5438. doi: 10.1128/IAI.72.9.5433-5438.2004

Fujimura, T., Anan, N., Sugimori, G., Watanabe, T., Jinushi, Y., Yoshida, I., et al. (2009). Susceptibility of *Pseudomonas aeruginosa* clinical isolates in Japan to doripenem and other antipseudomonal agents. *Int. J. Antimicrob. Agents* 34, 523–528. doi: 10.1016/j.ijantimicag.2009.07.008

Fung, D. K., Chan, E. W., Chin, M. L., and Chan, R. C. (2010). Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. *Antimicrob. Agents Chemother.* 54, 1082–1093. doi: 10.1128/AAC.01218-09

Furukawa, S., Kuchma, S. L., and O'Toole, G. A. (2006). Keeping their options open: acute versus persistent infections. *J. Bacteriol.* 188, 1211–1217. doi: 10.1128/JB.188.4.1211-1217.2006

García-Contreras, R. (2016). Is quorum sensing interference a viable alternative to treat *Pseudomonas aeruginosa* infections? *Front. Microbiol.* 7:1454. doi: 10.3389/fmicb.2016.01454

García-Contreras, R., Nuñez-López, L., Jasso-Chávez, R., Kwan, B. W., Belmont, J. A., Rangel-Vega, A., et al. (2015). Quorum sensing enhancement of the stress response promotes resistance to quorum quenching and prevents social cheating. *ISME J.* 9, 115–125. doi: 10.1038/ismej.2014.98

García-García, J. D., Sánchez-Thomas, R., and Moreno-Sánchez, R. (2016). Bio-recovery of non-essential heavy metals by intra- and extracellular mechanisms in free-living microorganisms. *Biotechnol. Adv.* 34, 859–873. doi: 10.1016/j.biotechadv.2016.05.003

Garneau, J. E., Dupuis, M. È., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., et al. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468, 67–71. doi: 10.1038/nature09523

Gellatly, S. L., and Hancock, R. E. W. (2013). *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathog. Dis.* 67, 159–173. doi: 10.1111/2049-632X.12033

Ghafoor, A., Hay, I. D., and Rehm, B. H. (2011). Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. *Appl. Environ. Microbiol.* 77, 5238–5246. doi: 10.1128/AEM.00637-11

Glick, J., and Garber, N. (1983). The intracellular localization of *Pseudomonas aeruginosa* lectins. *J. Gen. Microbiol.* 129, 3085–3090. doi: 10.1099/00221287-129-10-3085

Gloag, E. S., Turnbull, L., Huang, A., Vallotton, P., Wang, H., Nolan, L. M., et al. (2013). Self-organization of bacterial biofilms is facilitated by extracellular DNA. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11541–11546. doi: 10.1073/pnas.1218898110

González, J. E., and Keshavan, N. D. (2006). Messing with bacterial quorum sensing. *Microbiol. Mol. Biol. Rev.* 70, 859–875. doi: 10.1128/MMBR.00002-06

González-Valdez, A., Servín-González, L., Juarez, K., Hernandez-Aligio, A., and Soberón-Chávez, G. (2014). The effect of specific *rhlA-las*-box mutations on DNA binding and gene activation by *Pseudomonas aeruginosa* quorum-sensing transcriptional regulators RhlR and LasR. *FEMS Microbiol. Lett.* 356, 217–225. doi: 10.1111/1574-6968.12505

Grote, J., Krysciak, D., and Streit, W. R. (2015). Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogenous cell behavior. *Appl. Environ. Microbiol.* 81, 5280–5289. doi: 10.1128/AEM.00900-15

Gupta, K., Liao, J., Petrova, O. E., Cherny, K. E., and Sauer, K. (2014). Elevated levels of the second messenger c-di-GMP contribute to antimicrobial resistance of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 92, 488–506. doi: 10.1111/mmi.12587

Gutiérrez, O., Juan, C., Cercenado, E., Navarro, F., Bouza, E., Coll, P., et al. (2007). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. *Antimicrob. Agents Chemother.* 51, 4329–4335. doi: 10.1128/AAC.00810-07

Gutu, A. D., Sgambati, N., Strasbourger, P., Brannon, M. K., Jacobs, M. A., Haugen, E., et al. (2013). Polymyxin resistance of *Pseudomonas aeruginosa* phoQ mutants is dependent on additional two-component regulatory systems. *Antimicrob. Agents Chemother.* 57, 2204–2215. doi: 10.1128/AAC.02353-12

Guzzo, J., Pages, J. M., Duong, F., Lazdunski, A., and Murgier, M. (1991). *Pseudomonas aeruginosa* alkaline protease: evidence for secretion genes and study of secretion mechanism. *J. Bacteriol.* 173, 5290–5297. doi: 10.1128/jb.173.17.5290-5297.1991

Hall, S., McDermott, C., Anoopkumar-Dukie, S., McFarland, A. J., Forbes, A., Perkins, A. V., et al. (2016). Cellular effects of pyocyanin, a secreted virulence factor of *Pseudomonas aeruginosa*. *Toxins (Basel).* 8:236. doi: 10.3390/toxins8080236

Hammond, J. H., Dolben, E. F., Smith, T. J., Bhuju, S., and Hogan, D. A. (2015). Links between Anr and quorum sensing in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* 197, 2810–2820. doi: 10.1128/JB.00182-15

Hancock, R. E. W., and Speert, D. P. (2000). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resist Updat.* 3, 247–255. doi: 10.1054/drup.2000.0152

Hannauer, M., Braud, A., Hoegy, F., Ronot, P., Boos, A., and Schalk, I. J. (2012). The PvdRT-OpmQ efflux pump controls the metal selectivity of the iron uptake pathway mediated by the siderophore pyoverdine in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 14, 1696–1708. doi: 10.1111/j.1462-2920.2011.02674.x

Hassett, D. J. (1996). Anaerobic production of alginate by *Pseudomonas aeruginosa*: alginate restricts diffusion of oxygen. *J. Bacteriol.* 178, 7322–7325. doi: 10.1128/jb.178.24.7322-7325.1996

Hassett, D. J., Sutton, M. D., Schurr, M. J., Herr, A. B., Caldwell, C. C., and Matu, J. O. (2009). *Pseudomonas aeruginosa* hypoxic or anaerobic biofilm infections within cystic fibrosis airways. *Trends Microbiol.* 17, 130–138. doi: 10.1016/j.tim.2008.12.003

Häussler, S., Tümmler, B., Weissbrodt, H., Rohde, M., and Steinmetz, I. (1999). Small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. *Clin. Infect. Dis.* 29, 621–625. doi: 10.1086/598644

Häussler, S., Ziegler, I., Löttel, A., von Götz, F., Rohde, M., Wehmhöhner, D., et al. (2003). Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J. Med. Microbiol.* 52(Pt 4), 295–301. doi: 10.1099/jmm.0.05069-0

Hay, I. D., Gatland, K., Campisano, A., Jordens, J. Z., and Rehm, B. H. (2009a). Impact of alginate overproduction on attachment and biofilm architecture of

a supermucoid *Pseudomonas aeruginosa* strain. *Appl. Environ. Microbiol.* 75, 6022–6025. doi: 10.1128/AEM.01078-09

Hay, I. D., Remminghorst, U., and Rehm, B. H. (2009b). MucR, a novel membrane-associated regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 75, 1110–1120. doi: 10.1128/AEM.02416-08

Hay, I. D., Ur Rehman, Z., Ghafoor, A., and Rehm, B. H. (2010). Bacterial biosynthesis of alginates. *J. Chem. Techn. Biotechnol.* 85, 752–759. doi: 10.1002/jctb.2372

Hay, I. D., Ur Rehman, Z., Moradali, M. F., Wang, Y., and Rehm, B. H. (2013). Microbial alginate production, modification and its applications. *Microb. Biotechnol.* 6, 637–650. doi: 10.1111/1751-7915.12076

Hay, T., Fraud, S., Lau, C. H., Gilmour, C., and Poole, K. (2013). Antibiotic inducibility of the MexXY multidrug efflux operon of *Pseudomonas aeruginosa*: involvement of the MexZ anti-repressor ArmZ. *PLoS ONE* 8:e56858. doi: 10.1371/journal.pone.0056858

Henrichfreise, B., Wiegand, I., Pfister, W., and Wiedemann, B. (2007). Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. *Antimicrob. Agents Chemother.* 51, 4062–4070. doi: 10.1128/AAC.00148-07

Hershberger, C. D., Ye, R. W., Parsek, M. R., Xie, Z. D., and Chakrabarty, A. M. (1995). The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative sigma factor (sigma E). *Proc. Natl. Acad. Sci. U.S.A.* 92, 7941–7945. doi: 10.1073/pnas.92.17.7941

Hesketh, A., Chen, W. J., Ryding, J., Chang, S., and Bibb, M. (2007). The global role of ppGpp synthesis in morphological differentiation and antibiotic production in *Streptomyces coelicolor* A3(2). *Genome Biol.* 8:R161. doi: 10.1186/gb-2007-8-8-r161

Hickman, J. W., Tifrea, D. F., and Harwood, C. S. (2005). A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14422–14427. doi: 10.1073/pnas.0507170102

Higgins, P. G., Fluit, A. C., Milatovic, D., Verhoef, J., and Schmitz, F. J. (2003). Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 21, 409–413. doi: 10.1016/S0924-8579(03)00009-8

Hirai, K., Suzue, S., Irikura, T., Iyobe, S., and Mitsuhashi, S. (1987). Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 31, 582–586. doi: 10.1128/AAC.31.4.582

Hoffman, L. R., Kulasekara, H. D., Emerson, J., Houston, L. S., Burns, J. L., Ramsey, B. W., et al. (2009). *Pseudomonas aeruginosa lasR* mutants are associated with cystic fibrosis lung disease progression. *J. Cyst. Fibros.* 8, 66–70. doi: 10.1016/j.jcf.2008.09.006

Hoffman, L. R., Richardson, A. R., Houston, L. S., Kulasekara, H. D., Martens-Habbena, W., Klausen, M., et al. (2010). Nutrient availability as a mechanism for selection of antibiotic tolerant *Pseudomonas aeruginosa* within the CF airway. *PLoS Pathog.* 6:e1000712. doi: 10.1371/journal.ppat.1000712

Hogardt, M., and Heesemann, J. (2010). Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. *Int. J. Med. Microbiol.* 300, 557–562. doi: 10.1016/j.ijmm.2010.08.008

Hoge, R., Pelzer, A., Rosenau, F., and Wilhelm, S. (2010). Weapons of a pathogen: proteases and their role in virulence of *Pseudomonas aeruginosa*. *Current research, technology and education topics in applied microbiology and microbial biotechnology* 2, 383–395.

Hong, D. J., Bae, I. K., Jang, I. H., Jeong, S. H., Kang, H. K., and Lee, K. (2015). Epidemiology and characteristics of metallo-β-lactamase-producing *Pseudomonas aeruginosa*. *Infect Chemother.* 47, 81–97. doi: 10.3947/ic.2015.47.2.81

Imperi, F., Tiburzi, F., Fimia, G. M., and Visca, P. (2010). Transcriptional control of the *pvdS* iron starvation sigma factor gene by the master regulator of sulfur metabolism CysB in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 12, 1630–1642. doi: 10.1111/j.1462-2920.2010.02210.x

Irie, Y., Starkey, M., Edwards, A. N., Wozniak, D. J., Romeo, T., and Parsek, M. R. (2010). *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol. Microbiol.* 78, 158–172. doi: 10.1111/j.1365-2958.2010.07320.x

Jaffar-Bandjee, M. C., Lazdunski, A., Bally, M., Carrere, J., Chazalette, J. P., and Galabert, C. (1995). Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 33, 924–929.

Jeannot, K., Elsen, S., Kohler, T., Attree, I., van Delden, C., and Plésiat, P. (2008). Resistance and virulence of *Pseudomonas aeruginosa* clinical strains overproducing the MexCD-OprJ efflux pump. *Antimicrob. Agents Chemother.* 52, 2455–2462. doi: 10.1128/AAC.01107-07

Jennings, L. K., Storek, K. M., Ledvina, H. E., Coulon, C., Marmont, L. S., Sadovskaya, I., et al. (2015). Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. *Proc. Natl. Acad. Sci. U.S.A.* 112, 11353–11358. doi: 10.1073/pnas.1503058112

Jensen, P. Ø., Bjarnsholt, T., Phipps, R., Rasmussen, T. B., Calum, H., Christoffersen, L., et al. (2007). Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology* 153(Pt 5), 1329–1338. doi: 10.1099/mic.0.2006/003863-0

Jimenez, P. N., Koch, G., Thompson, J. A., Xavier, K. B., Cool, R. H., and Quax, W. J. (2012). The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.* 76, 46–65. doi: 10.1128/MMBR.05007-11

Jiricny, N., Molin, S., Foster, K., Diggle, S. P., Scanlan, P. D., Ghoul, M., et al. (2014). Loss of social behaviours in populations of *Pseudomonas aeruginosa* infecting lungs of patients with cystic fibrosis. *PLoS ONE* 9:e83124. doi: 10.1371/journal.pone.0083124

Johnson, A. P., and Woodford, N. (2013). Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. *J Med. Microbiol.* 62(Pt 4), 499–513. doi: 10.1099/jmm.0.052555-0

Jones, P., Palser, S. C., Pravle, A. P., Hurley, M. N., and Smyth, A. R. (2016). WS20.1 Secular trends in *Pseudomonas aeruginosa* acquisition in the United Kingdom: a registry study. *J. Cyst. Fibros* 15(Suppl. 1), S31. doi: 10.1016/S1569-1993(16)30169-2

Juan, C., Macia, M. D., Gutierrez, O., Vidal, C., Perez, J. L., and Oliver, A. (2005). Molecular mechanisms of β-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. *Antimicrob. Agents Chemother.* 49, 4733–4738. doi: 10.1128/AAC.49.11.4733-4738.2005

Jyot, J., and Ramphal, R. (2008). "Flagella and pili of *Pseudomonas aeruginosa*," in *Pseudomonas: Model Organism, Pathogen, Cell Factory,* ed B. H. A. Rehm (Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA), 85–108.

Kao, C. Y., Chen, S. S., Hung, K. H., Wu, H. M., Hsueh, P. R., Yan, J. J., et al. (2016). Overproduction of active efflux pump and variations of OprD dominate in imipenem-resistant *Pseudomonas aeruginosa* isolated from patients with bloodstream infections in Taiwan. *BMC Microbiol.* 16:107. doi: 10.1186/s12866-016-0719-2

Karatuna, O., and Yagci, A. (2010). Analysis of quorum sensing-dependent virulence factor production and its relationship with antimicrobial susceptibility in *Pseudomonas aeruginosa* respiratory isolates. *Clin. Microbiol. Infect.* 16, 1770–1775. doi: 10.1111/j.1469-0691.2010.03177.x

Karlowsky, J. A., Hoban, D. J., Zelenitsky, S. A., and Zhanel, G. G. (1997). Altered *denA* and *anr* gene expression in aminoglycoside adaptive resistance in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 40, 371–376. doi: 10.1093/jac/40.3.371

Kenward, M. A., Brown, M. R., Hesslewood, S. R., and Dillon, C. (1978). Influence of R-plasmid RP1 of *Pseudomonas aeruginosa* on cell wall composition, drug resistance, and sensitivity to cold shock. *Antimicrob. Agents Chemother.* 13, 446–453. doi: 10.1128/AAC.13.3.446

Kessler, E., Safrin, M., Abrams, W. R., Rosenbloom, J., and Ohman, D. E. (1997). Inhibitors and specificity of *Pseudomonas aeruginosa* LasA. *J. Biol. Chem.* 272, 9884–9889. doi: 10.1074/jbc.272.15.9884

Khaledi, A., Schniederjans, M., Pohl, S., Rainer, R., Bodenhofer, U., Xia, B., et al. (2016). Transcriptome profiling of antimicrobial resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 60, 4722–4733. doi: 10.1128/AAC.00075-16

Khan, T. Z., Wagener, J. S., Bost, T., Martinez, J., Accurso, F. J., and Riches, D. W. H. (1995). Early pulmonary inflammation in infants with cystic fibrosis. *Am. J. Resp. Crit. Care* 151, 1075–1082.

Kim, E. J., Sabra, W., and Zeng, A. P. (2003). Iron deficiency leads to inhibition of oxygen transfer and enhanced formation of virulence factors in cultures of *Pseudomonas aeruginosa* PAO1. *Microbiology* 149(Pt 9), 2627–2634. doi: 10.1099/mic.0.26276-0

Kim, H. Y., Schlictman, D., Shankar, S., Xie, Z., Chakrabarty, A. M., and Kornberg, A. (1998). Alginate, inorganic polyphosphate, GTP and ppGpp synthesis co-regulated in *Pseudomonas aeruginosa*: implications for stationary phase survival and synthesis of RNA/DNA precursors. *Mol. Microbiol.* 27, 717–725. doi: 10.1046/j.1365-2958.1998.00702.x

Kirisits, M. J., Prost, L., Starkey, M., and Parsek, M. R. (2005). Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 71, 4809–4821. doi: 10.1128/AEM.71.8.4809-4821.2005

Kohanski, M. A., Dwyer, D. J., Wierzbowski, J., Cottarel, G., and Collins, J. J. (2008). Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. *Cell* 135, 679–690. doi: 10.1016/j.cell.2008.09.038

Köhler, T., Michéa-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L. K., and Pechère, J. C. (1997). Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 23, 345–354. doi: 10.1046/j.1365-2958.1997.2281594.x

Köhler, T., van Delden, C., Curty, L. K., Hamzehpour, M. M., and Pechere, J. C. (2001). Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. *J. Bacteriol.* 183, 5213–5222. doi: 10.1128/JB.183.18.5213-5222.2001

Kong, K.-F., Jayawardena, S. R., Indulkar, S. D., del Puerto, A., Koh, C.-L., Høiby, N., et al. (2005). *Pseudomonas aeruginosa* AmpR is a global transcriptional factor that regulates expression of AmpC and PoxB β-lactamases, proteases, quorum sensing, and other virulence factors. *Antimicrob. Agents Chemother.* 49, 4567–4575. doi: 10.1128/AAC.49.11.4567-4575.2005

Kung, V. L., Ozer, E. A., and Hauser, A. R. (2010). The accessory genome of *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.* 74, 621–641. doi: 10.1128/MMBR.00027-10

Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., et al. (2001). Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* 293, 705–708. doi: 10.1126/science.1061315

Laarman, A. J., Bardoel, B. W., Ruyken, M., Fernie, J., Milder, F. J., van Strijp, J. A., et al. (2012). *Pseudomonas aeruginosa* alkaline protease blocks complement activation via the classical and lectin pathways. *J. Immunol.* 188, 386–393. doi: 10.4049/jimmunol.1102162

LaFayette, S. L., Houle, D., Beaudoin, T., Wojewodka, G., Radzioch, D., Hoffman, L. R., et al. (2015). Cystic fibrosis-adapted *Pseudomonas aeruginosa* quorum sensing *lasR* mutants cause hyperinflammatory responses. *Sci. Adv.* 1:e1500199. doi: 10.1126/sciadv.1500199

Lamarche, M. G., and Déziel, E. (2011). MexEF-OprN efflux pump exports the *Pseudomonas* quinolone signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). *PLoS ONE* 6:e24310. doi: 10.1371/journal.pone.0024310

Lambert, M. L., Suetens, C., Savey, A., Palomar, M., Hiesmayr, M., Morales, I., et al. (2011). Clinical outcomes of health-care-associated infections and antimicrobial resistance in patients admitted to European intensive-care units: a cohort study. *Lancet Infect. Dis.* 11, 30–38. doi: 10.1016/S1473-3099(10)70258-9

Lapouge, K., Schubert, M., Allain, F. H., and Haas, D. (2008). Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol. Microbiol.* 67, 241–253. doi: 10.1111/j.1365-2958.2007.06042.x

LaSarre, B., and Federle, M. J. (2013). Exploiting quorum sensing to confuse bacterial pathogens. *Microbiol. Mol. Biol. Rev.* 77, 73–111. doi: 10.1128/MMBR.00046-12

Lau, C. H., Hughes, D., and Poole, K. (2014). MexY-promoted aminoglycoside resistance in *Pseudomonas aeruginosa*: involvement of a putative proximal binding pocket in aminoglycoside recognition. *Mbio* 5:e01068. doi: 10.1128/mBio.01068-14

Laughlin, R. S., Musch, M. W., Hollbrook, C. J., Rocha, F. M., Chang, E. B., and Alverdy, J. C. (2000). The key role of *Pseudomonas aeruginosa* PA-I lectin on experimental gut-derived sepsis. *Ann. Surg.* 232, 133–142. doi: 10.1097/00000658-200007000-00019

Lee, J. K., Lee, Y. S., Park, Y. K., and Kim, B. S. (2005). Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 25, 290–295. doi: 10.1016/j.ijantimicag.2004.11.012

Lee, J., Wu, J., Deng, Y., Wang, J., Wang, C., Wang, J., et al. (2013). A cell-cell communication signal integrates quorum sensing and stress response. *Nat. Chem. Biol.* 9, 339–343. doi: 10.1038/nchembio.1225

Lee, J. Y., Park, Y. K., Chung, E. S., Na, I. Y., and Ko, K. S. (2016). Evolved resistance to colistin and its loss due to genetic reversion in *Pseudomonas aeruginosa*. *Sci. Rep.* 6:25543. doi: 10.1038/srep25543

Lee, J., and Zhang, L. (2015). The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell* 6, 26–41. doi: 10.1007/s13238-014-0100-x

Leid, J. G. (2009). Bacterial biofilms resist key host defenses. *Microbe* 4, 66–70.

Lenney, W., and Gilchrist, F. J. (2011). *Pseudomonas aeruginosa* and cyanide production. *Eur. Respir. J.* 37, 482–483. doi: 10.1183/09031936.00122810

Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nat. Rev. Microbiol.* 5, 48–56. doi: 10.1038/nrmicro1557

Lewis, K. (2008). Multidrug tolerance of biofilms and persister cells. *Curr. Top. Microbiol. Immunol.* 322, 107–131. doi: 10.1007/978-3-540-75418-3_6

Li, G., Shen, M., Lu, S., Le, S., Tan, Y., Wang, J., et al. (2016). Identification and characterization of the HicAB toxin-antitoxin system in the opportunistic pathogen *Pseudomonas aeruginosa*. *Toxins (Basel).* 8:113. doi: 10.3390/toxins8040113

Li, M., Long, Y., Liu, Y., Liu, Y., Chen, R., Shi, J., et al. (2016). HigB of *Pseudomonas aeruginosa* enhances killing of phagocytes by up-regulating the type III secretion system in ciprofloxacin induced persister cells. *Front. Cell. Infect. Microbiol.* 6:125. doi: 10.3389/fcimb.2016.00125

Li, X. Z., Plésiat, P., and Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin. Microbiol. Rev.* 28, 337–418. doi: 10.1128/CMR.00117-14

Li, Y. Q., Du, X. L., Lu, Z. J., Wu, D. Q., Zhao, Y. L., Ren, B., et al. (2011). Regulatory feedback loop of two *phz* gene clusters through 5′-untranslated regions in *Pseudomonas* sp M18. *PLoS ONE* 6:e19413. doi: 10.1371/journal.pone.0019413

Lister, P. D., Wolter, D. J., and Hanson, N. D. (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin. Microbiol. Rev.* 22, 582–610. doi: 10.1128/CMR.00040-09

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/S1473-3099(15)00424-7

Llanes, C., Kohler, T., Patry, I., Dehecq, B., van Delden, C., and Plésiat, P. (2011). Role of the MexEF-OprN efflux system in low-level resistance of *Pseudomonas aeruginosa* to ciprofloxacin. *Antimicrob. Agents Chemother.* 55, 5676–5684. doi: 10.1128/AAC.00101-11

Long, J., Zaborina, O., Holbrook, C., Zaborin, A., and Alverdy, J. (2008). Depletion of intestinal phosphate after operative injury activates the virulence of *P. aeruginosa* causing lethal gut-derived sepsis. *Surgery* 144, 189–197. doi: 10.1016/j.surg.2008.03.045

Louwen, R., Staals, R. H. J., Endtz, H. P., van Baarlen, P., and van der Oost, J. (2014). The role of CRISPR-Cas systems in virulence of pathogenic bacteria. *Microbiol. Mol. Biol. Rev.* 78, 74–88. doi: 10.1128/MMBR.00039-13

Ma, L., Conover, M., Lu, H., Parsek, M. R., Bayles, K., and Wozniak, D. J. (2009). Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog.* 5:e1000354. doi: 10.1371/journal.ppat.1000354

MacDougall, C., Harpe, S. E., Powell, J. P., Johnson, C. K., Edmond, M. B., and Polk, R. E. (2005). *Pseudomonas aeruginosa, Staphylococcus aureus*, and fluoroquinolone use. *Emerging Infect. Dis.* 11, 1197–1204. doi: 10.3201/eid1108.050116

Mahenthiralingam, E., Campbell, M. E., and Speert, D. P. (1994). Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect. Immun.* 62, 596–605.

Maisonneuve, E., Castro-Camargo, M., and Gerdes, K. (2013). (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. *Cell* 154, 1140–1150. doi: 10.1016/j.cell.2013.07.048

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J. J., Charpentier, E., Horvath, P., et al. (2011). Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol.* 9, 467–477. doi: 10.1038/nrmicro2577

Malone, J. G. (2015). Role of small colony variants in persistence of *Pseudomonas aeruginosa* infections in cystic fibrosis lungs. *Infect. Drug Resist.* 8, 237–247. doi: 10.2147/IDR.S68214

Malone, J. G., Jaeger, T., Manfredi, P., Dötsch, A., Blanka, A., Bos, R., et al. (2012). The YfiBNR signal transduction mechanism reveals novel targets for the evolution of persistent *Pseudomonas aeruginosa* in cystic fibrosis airways. *PLoS Pathog.* 8:e1002760. doi: 10.1371/journal.ppat.1002760

Mann, E. E., and Wozniak, D. J. (2012). *Pseudomonas* biofilm matrix composition and niche biology. *FEMS Microbiol. Rev.* 36, 893–916. doi: 10.1111/j.1574-6976.2011.00322.x

Marr, A. K., Overhage, J., Bains, M., and Hancock, R. E. W. (2007). The Lon protease of *Pseudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility. *Microbiology* 153(Pt 2), 474–482. doi: 10.1099/mic.0.2006/002519-0

Martinez, J. L., Sánchez, M. B., Martínez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A., et al. (2009). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol. Rev.* 33, 430–449. doi: 10.1111/j.1574-6976.2008.00157.x

Marvig, R. L., Sommer, L. M., Molin, S., and Johansen, H. K. (2015). Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat. Genet.* 47, 57–64. doi: 10.1038/ng.3148

Maseda, H., Saito, K., Nakajima, A., and Nakae, T. (2000). Variation of the *mexT* gene, a regulator of the MexEF-oprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* 192, 107–112. doi: 10.1111/j.1574-6968.2000.tb09367.x

Maseda, H., Uwate, M., and Nakae, T. (2010). Transcriptional regulation of the MexEF-oprN multidrug efflux pump operon by MexT and an unidentified repressor in *nfxC*-type mutant of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* 311, 36–43. doi: 10.1111/j.1574-6968.2010.02063.x

Mashburn, L. M., and Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437, 422–425. doi: 10.1038/nature03925

Mataseje, L. F., Peirano, G., Church, D. L., Conly, J., Mulvey, M., and Pitout, J. D. (2016). Colistin-nonsusceptible *Pseudomonas aeruginosa* sequence type 654 with bla$_{NDM-1}$ arrives in North America. *Antimicrob. Agents Chemother.* 60, 1794–1800. doi: 10.1128/AAC.02591-15

Mathee, K., Ciofu, O., Sternberg, C., Lindum, P. W., Campbell, J. I., Jensen, P., et al. (1999). Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology* 145(Pt 6), 1349–1357. doi: 10.1099/13500872-145-6-1349

Mathee, K., Narasimhan, G., Valdes, C., Qiu, X., Matewish, J. M., Koehrsen, M., et al. (2008). Dynamics of *Pseudomonas aeruginosa* genome evolution. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3100–3105. doi: 10.1073/pnas.0711982105

Matsuo, Y., Eda, S., Gotoh, N., Yoshihara, E., and Nakae, T. (2004). MexZ-mediated regulation of MexXY multidrug efflux pump expression in Pseudomonas aeruginosa by binding on the *mexZ-mexX* intergenic DNA. *FEMS Microbiol. Lett.* 238, 23–28. doi: 10.1016/j.femsle.2004.07.010

Maxwell, K. L. (2016). Phages fight back: inactivation of the CRISPR-Cas bacterial immune system by anti-CRISPR proteins. *PLoS Pathog.* 12:e1005282. doi: 10.1371/journal.ppat.1005282

McDaniel, C. T., Panmanee, W., and Hassett, D. J. (2015). "An overview of infections in cystic fibrosis airways and the role of environmental conditions on Pseudomonas aeruginosa biofilm formation and viability," in *Cystic Fibrosis in the Light of New Research*, ed D. Wat (InTech), 171–198.

Meletis, G., and Bagkeri, M. (2013). "Pseudomonas aeruginosa: multi-drug-resistance development and treatment options," in *Infection Control,* ed S. Basak (InTech), 33–56.

Michalska, M., and Wolf, P. (2015). *Pseudomonas* Exotoxin A: optimized by evolution for effective killing. *Front. Microbiol.* 6:963. doi: 10.3389/fmicb.2015.00963

Mishra, M., Byrd, M. S., Sergeant, S., Azad, A. K., Parsek, M. R., McPhail, L., et al. (2012). *Pseudomonas aeruginosa* Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. *Cell. Microbiol.* 14, 95–106. doi: 10.1111/j.1462-5822.2011.01704.x

Mojica, F. J., Díez-Villaseñor, C., García-Martínez, J., and Almendros, C. (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 155(Pt 3), 733–740. doi: 10.1099/mic.0.023960-0

Moradali, M. F., Donati, I., Sims, I. M., Ghods, S., and Rehm, B. H. (2015). Alginate polymerization and modification are linked in *Pseudomonas aeruginosa*. *MBio* 6, e00453–e00415. doi: 10.1128/mBio.00453-15

Moscoso, J. A., Mikkelsen, H., Heeb, S., Williams, P., and Filloux, A. (2011). The *Pseudomonas aeruginosa* sensor RetS switches type III and type VI secretion via c-di-GMP signalling. *Environ. Microbiol.* 13, 3128–3138. doi: 10.1111/j.1462-2920.2011.02595.x

Moskowitz, S. M., Brannon, M. K., Dasgupta, N., Pier, M., Sgambati, N., Miller, A. K., et al. (2012). PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob. Agents Chemother.* 56, 1019–1030. doi: 10.1128/AAC.05829-11

Mouneimné, H., Robert, J., Jarlier, V., and Cambau, E. (1999). Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 43, 62–66.

Mulcahy, H., Charron-Mazenod, L., and Lewenza, S. (2008). Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog.* 4:e1000213. doi: 10.1371/journal.ppat.1000213

Mulcahy, L. R., Burns, J. L., Lory, S., and Lewis, K. (2010). Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J. Bacteriol.* 192, 6191–6199. doi: 10.1128/JB.01651-09

Nathwani, D., Raman, G., Sulham, K., Gavaghan, M., and Menon, V. (2014). Clinical and economic consequences of hospital-acquired resistant and multidrug-resistant *Pseudomonas aeruginosa* infections: a systematic review and meta-analysis. *Antimicrob. Resist. Infect. Control* 3:32. doi: 10.1186/2047-2994-3-32

Nelson, L. K., D'Amours, G. H., Sproule-Willoughby, K. M., Morck, D. W., and Ceri, H. (2009). *Pseudomonas aeruginosa* las and rhl quorum-sensing systems are important for infection and inflammation in a rat prostatitis model. *Microbiology* 155(Pt 8), 2612–2619. doi: 10.1099/mic.0.028464-0

Nguyen, A. T., Jones, J. W., Camara, M., Williams, P., Kane, M. A., and Oglesby-Sherrouse, A. G. (2016). Cystic fibrosis isolates of *Pseudomonas aeruginosa* retain iron-regulated antimicrobial activity against *Staphylococcus aureus* through the action of multiple alkylquinolones. *Front. Microbiol.* 7:1171. doi: 10.3389/fmicb.2016.01171

Nguyen, A. T., Jones, J. W., Ruge, M. A., Kane, M. A., and Oglesby-Sherrouse, A. G. (2015). Iron depletion enhances production of antimicrobials by *Pseudomonas aeruginosa*. *J. Bacteriol.* 197, 2265–2275. doi: 10.1128/JB.00072-15

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., et al. (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* 334, 982–986. doi: 10.1126/science.1211037

Ochs, M. M., McCusker, M. P., Bains, M., and Hancock, R. E. (1999). Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. *Antimicrob. Agents Chemother.* 43, 1085–1090.

Oldak, E., and Trafny, E. A. (2005). Secretion of proteases by *Pseudomonas aeruginosa* biofilms exposed to ciprofloxacin. *Antimicrob. Agents Chemother.* 49, 3281–3288. doi: 10.1128/AAC.49.8.3281-3288.2005

Oliver, A., Cantón, R., Campo, P., Baquero, F., and Blázquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288, 1251–1253. doi: 10.1126/science.288.5469.1251

Oliver, A., and Mena, A. (2010). Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. *Clin. Microbiol. Infect.* 16, 798–808. doi: 10.1111/j.1469-0691.2010.03250.x

Oliver, A., Mulet, X., López-Causapé, C., and Juan, C. (2015). The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist. Updat.* 21–22, 41–59. doi: 10.1016/j.drup.2015.08.002

Olsen, I. (2015). Biofilm-specific antibiotic tolerance and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 877–886. doi: 10.1007/s10096-015-2323-z

Overhage, J., Schemionek, M., Webb, J. S., and Rehm, B. H. (2005). Expression of the psl operon in Pseudomonas aeruginosa PAO1 biofilms: PslA performs an essential function in biofilm formation. *Appl. Environ. Microbiol.* 71, 4407–4413. doi: 10.1128/AEM.71.8.4407-4413.2005

Ozer, E. A., Allen, J. P., and Hauser, A. R. (2014). Characterization of the core and accessory genomes of *Pseudomonas aeruginosa* using bioinformatic tools Spine and AGEnt. *BMC Genomics* 15:737. doi: 10.1186/1471-2164-15-737

Pagedar, A., Singh, J., and Batish, V. K. (2011). Efflux mediated adaptive and cross resistance to ciprofloxacin and benzalkonium chloride in *Pseudomonas aeruginosa* of dairy origin. *J. Basic Microbiol.* 51, 289–295. doi: 10.1002/jobm.201000292

Palmer, K. L., and Whiteley, M. (2011). DMS3-42: the secret to CRISPR-dependent biofilm inhibition in *Pseudomonas aeruginosa*. *J. Bacteriol.* 193, 3431–3432. doi: 10.1128/JB.05066-11

Pandey, D. P., and Gerdes, K. (2005). Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res.* 33, 966–976. doi: 10.1093/nar/gki201

Papenfort, K., and Bassler, B. L. (2016). Quorum sensing signal-response systems in Gram-negative bacteria. *Nat. Rev. Microbiol.* 14, 576–588. doi: 10.1038/nrmicro.2016.89

Paterson, D. L., and Bonomo, R. A. (2005). Extended-spectrum β-lactamases: a clinical update. *Clin. Microbiol. Rev.* 18, 657–686. doi: 10.1128/CMR.18.4.657-686.2005

Paul, K., Nieto, V., Carlquist, W. C., Blair, D. F., and Harshey, R. M. (2010). The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a "backstop brake" mechanism. *Mol. Cell* 38, 128–139. doi: 10.1016/j.molcel.2010.03.001

Pawluk, A., Bondy-Denomy, J., Cheung, V. H. W., Maxwell, K. L., and Davidson, A. R. (2014). A new group of phage Anti-CRISPR genes inhibits the type I-E CRISPR-Cas system of *Pseudomonas aeruginosa*. *MBio* 5:e00896-14. doi: 10.1128/mBio.00896-14

Pessi, G., and Haas, D. (2000). Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J. Bacteriol.* 182, 6940–6949. doi: 10.1128/JB.182.24.6940-6949.2000

Petrova, O. E., and Sauer, K. (2009). A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. *PLoS Pathog.* 5:e1000668. doi: 10.1371/journal.ppat.1000668

Pillar, C. M., and Hobden, J. A. (2002). *Pseudomonas aeruginosa* exotoxin A and keratitis in mice. *Invest. Ophthalmol. Vis. Sci.* 43, 1437–1444. Available online at: http://iovs.arvojournals.org/article.aspx?articleid=2123690

Poole, K. (2005). Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 49, 479–487. doi: 10.1128/AAC.49.2.479-487.2005

Poole, K. (2011). *Pseudomonas aeruginosa*: resistance to the max. *Front. Microbiol.* 2:65. doi: 10.3389/fmicb.2011.00065

Poole, K. (2012). Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. *Trends Microbiol.* 20, 227–234. doi: 10.1016/j.tim.2012.02.004

Porter, S. L., Wadhams, G. H., and Armitage, J. P. (2011). Signal processing in complex chemotaxis pathways. *Nat. Rev. Microbiol.* 9, 153–165. doi: 10.1038/nrmicro2505

Potron, A., Poirel, L., and Nordmann, P. (2015). Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int. J. Antimicrob. Agents* 45, 568–585. doi: 10.1016/j.ijantimicag.2015.03.001

Potrykus, K., and Cashel, M. (2008). (p)ppGpp: still magical? *Annu. Rev. Microbiol.* 62, 35–51. doi: 10.1146/annurev.micro.62.081307.162903

Pourmand, M., Sadighian, H., and Naderi, M. (2015). Relation between expression of the *las* quorum-sensing system in clinical isolates of *Pseudomonas aeruginosa* and expression of efflux pump and *ampC*. *J. Med. Bacteriol.* 2, 32–40. Available online at: http://tums.ac.ir/1393/04/18/Sadighian.pdf-mpourmand-2014-07-09-06-33.pdf

Quale, J., Bratu, S., Gupta, J., and Landman, D. (2006). Interplay of efflux system, *ampC*, and *oprD* expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother.* 50, 1633–1641. doi: 10.1128/AAC.50.5.1633-1641.2006

Ramisetty, B. C. M., Ghosh, D., Chowdhury, M. R., and Santhosh, R. S. (2016). What is the link between stringent response, endoribonuclease encoding Type II Toxin-Antitoxin systems and persistence? *bioRxiv*. *Front. Microbiol.* 7:1882. doi: 10.1101/069831

Ramisse, F., van Delden, C., Gidenne, S., Cavallo, J., and Hernandez, E. (2000). Decreased virulence of a strain of *Pseudomonas aeruginosa* O12 overexpressing a chromosomal type 1 β-lactamase could be due to reduced expression of cell-to-cell signaling dependent virulence factors. *FEMS Immunol. Med. Microbiol.* 28, 241–245. doi: 10.1111/j.1574-695X.2000.tb01483.x

Rasamiravaka, T., and El Jaziri, M. (2016). Quorum-sensing mechanisms and bacterial response to antibiotics in *P. aeruginosa*. *Curr Microbiol.* doi: 10.1007/s00284-016-1101-1

Rau, M. H., Hansen, S. K., Johansen, H. K., Thomsen, L. E., Workman, C. T., Nielsen, K. F., et al. (2010). Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. *Environ. Microbiol.* 12, 1643–1658. doi: 10.1111/j.1462-2920.2010.02211.x

Rehm, B. H., Grabert, E., Hein, J., and Winkler, U. K. (1994). Antibody response of rabbits and cystic fibrosis patients to an alginate-specific outer membrane protein of a mucoid strain of *Pseudomonas aeruginosa*. *Microb. Pathog.* 16, 43–51. doi: 10.1006/mpat.1994.1004

Remminghorst, U., and Rehm, B. H. (2006). Alg44, a unique protein required for alginate biosynthesis in Pseudomonas aeruginosa. *FEBS Lett.* 580, 3883–3888. doi: 10.1016/j.febslet.2006.05.077

Rocker, A., and Meinhart, A. (2016). Type II toxin: antitoxin systems. More than small selfish entities? *Curr. Genet.* 62, 287–290. doi: 10.1007/s00294-015-0541-7

Rojas Murcia, N., Lee, X., Waridel, P., Maspoli, A., Imker, H. J., Chai, T., et al. (2015). The *Pseudomonas aeruginosa* antimetabolite L -2-amino-4-methoxy-trans-3-butenoic acid (AMB) is made from glutamate and two alanine residues via a thiotemplate-linked tripeptide precursor. *Front. Microbiol.* 6:170. doi: 10.3389/fmicb.2015.00170

Römling, U., and Balsalobre, C. (2012). Biofilm infections, their resilience to therapy and innovative treatment strategies. *J. Intern. Med.* 272, 541–561. doi: 10.1111/joim.12004

Römling, U., Galperin, M. Y., and Gomelsky, M. (2013). Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* 77, 1–52. doi: 10.1128/MMBR.00043-12

Rosenfeld, M., Gibson, R. L., McNamara, S., Emerson, J., Burns, J. L., Castile, R., et al. (2001). Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. *Pediatr. Pulmonol.* 32, 356–366. doi: 10.1002/ppul.1144

Rosenthal, V. D., Al-Abdely, H. M., El-Kholy, A. A., AlKhawaja, S. A. A., Leblebicioglu, H., Mehta, Y., et al. (2016). International nosocomial infection control consortium report, data summary of 50 countries for 2010-2015: device-associated module. *Am. J. Infect. Control.* 44, 1495–1504. doi: 10.1016/j.ajic.2016.08.007

Russotto, V., Cortegiani, A., Raineri, S. M., and Giarratano, A. (2015). Bacterial contamination of inanimate surfaces and equipment in the intensive care unit. *J. Intensive Care* 3, 54. doi: 10.1186/s40560-015-0120-5

Ryan, R. P., Fouhy, Y., Lucey, J. F., and Dow, J. M. (2006). Cyclic di-GMP signaling in bacteria: recent advances and new puzzles. *J. Bacteriol.* 188, 8327–8334. doi: 10.1128/JB.01079-06

Sampathkumar, G., Khakimova, M., Chan, T., and Nguyen, D. (2016). "The stringent response and antioxidant defences in *Pseudomonas aeruginosa*," in *Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria*, ed F. J. de Bruijn (Hoboken, NJ: John Wiley & Sons, Inc.), 500–506.

Schafhauser, J., Lepine, F., McKay, G., Ahlgren, H. G., Khakimova, M., and Nguyen, D. (2014). The stringent response modulates 4-hydroxy-2-alkylquinoline biosynthesis and quorum-sensing hierarchy in *Pseudomonas aeruginosa*. *J. Bacteriol.* 196, 1641–1650. doi: 10.1128/JB.01086-13

Schalk, I. J., and Guillon, L. (2013). Pyoverdine biosynthesis and secretion in *Pseudomonas aeruginosa*: implications for metal homeostasis. *Environ. Microbiol.* 15, 1661–1673. doi: 10.1111/1462-2920.12013

Schmiel, D. H., and Miller, V. L. (1999). Bacterial phospholipases and pathogenesis. *Microbes Infect* 1, 1103–1112. doi: 10.1016/S1286-4579(99)00205-1

Schneider, M., Mühlemann, K., Droz, S., Couzinet, S., Casaulta, C., and Zimmerli, S. (2008). Clinical characteristics associated with isolation of small-colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. *J. Clin. Microbiol.* 46, 1832–1834. doi: 10.1128/JCM.00361-08

Schroder, K., Hertzog, P. J., Ravasi, T., and Hume, D. A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 75, 163–189. doi: 10.1189/jlb.0603252

Schurr, M. J., Yu, H., Martinez-Salazar, J. M., Boucher, J. C., and Deretic, V. (1996). Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *J. Bacteriol.* 178, 4997–5004. doi: 10.1128/jb.178.16.4997-5004.1996

Shih, P. C., and Huang, C. T. (2002). Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance. *J. Antimicrob. Chemother.* 49, 309–314. doi: 10.1093/jac/49.2.309

Slater, H., Crow, M., Everson, L., and Salmond, G. P. (2003). Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in Serratia via both quorum-sensing-dependent and -independent pathways. *Mol. Microbiol.* 47, 303–320. doi: 10.1046/j.1365-2958.2003.03295.x

Sobel, M. L., Hocquet, D., Cao, L., Plesiat, P., and Poole, K. (2005). Mutations in PA3574 (nalD) lead to increased MexAB-OprM expression and multidrug resistance in laboratory and clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 49, 1782–1786. doi: 10.1128/AAC.49.5.1782-1786.2005

Starkey, M., Hickman, J. H., Ma, L. Y., Zhang, N., De Long, S., Hinz, A., et al. (2009). *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *J. Bacteriol.* 191, 3492–3503. doi: 10.1128/JB.00119-09

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., et al. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959–964. doi: 10.1038/35023079

Strateva, T., and Yordanov, D. (2009). *Pseudomonas aeruginosa* - a phenomenon of bacterial resistance. *J. Med. Microbiol.* 58, 1133–1148. doi: 10.1099/jmm.0.009142-0

Strempel, N., Neidig, A., Nusser, M., Geffers, R., Vieillard, J., Lesouhaitier, O., et al. (2013). Human host defense peptide LL-37 stimulates virulence factor production and adaptive resistance in *Pseudomonas aeruginosa*. *PLoS ONE* 8:e82240. doi: 10.1371/journal.pone.0082240

Sullivan, R., Schaus, D., John, M., and Delport, J. A. (2016). Extended spectrum beta-lactamases: a minireview of clinical relevant groups. *J. Med. Microb. Diagn.* 4:203. doi: 10.4172/2161-0703.1000203

Sun, J., Deng, Z., and Yan, A. (2014). Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochem. Biophys. Res. Commun.* 453, 254–267. doi: 10.1016/j.bbrc.2014.05.090

Sun, S., Zhou, L., Jin, K., Jiang, H., and He, Y. W. (2016). Quorum sensing systems differentially regulate the production of phenazine-1-carboxylic acid in the rhizobacterium *Pseudomonas aeruginosa* PA1201. *Sci. Rep.* 6:30352. doi: 10.1038/srep30352

Tamber, S., and Hancock, R. E. (2003). On the mechanism of solute uptake in *Pseudomonas*. *Front. Biosci.* 8:s472–s483. doi: 10.2741/1075

Tian, Z. X., Mac Aogain, M., O'Connor, H. F., Fargier, E., Mooij, M. J., Adams, C., et al. (2009). MexT modulates virulence determinants in *Pseudomonas aeruginosa* independent of the MexEF-OprN efflux pump. *Microb. Pathog.* 47, 237–241. doi: 10.1016/j.micpath.2009.08.003

Tielen, P., Strathmann, M., Jaeger, K. E., Flemming, H. C., and Wingender, J. (2005). Alginate acetylation influences initial surface colonization by mucoid *Pseudomonas aeruginosa*. *Microbiol. Res.* 160, 165–176. doi: 10.1016/j.micres.2004.11.003

Toder, D. S., Ferrell, S. J., Nezezon, J. L., Rust, L., and Iglewski, B. H. (1994). *lasA* and *lasB* genes of *Pseudomonas aeruginosa* - Analysis of transcription and gene product activity. *Infect. Immun.* 62, 1320–1327.

Toder, D. S., Gambello, M. J., and Iglewski, B. H. (1991). *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. *Mol. Microbiol.* 5, 2003–2010. doi: 10.1111/j.1365-2958.1991.tb00822.x

Trejo-Hernández, A., Andrade-Domínguez, A., Hernández, M., and Encarnación, S. (2014). Interspecies competition triggers virulence and mutability in *Candida albicans-Pseudomonas aeruginosa* mixed biofilms. *ISME J.* 8, 1974–1988. doi: 10.1038/ismej.2014.53

Trias, J., and Nikaido, H. (1990). Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 34, 52–57. doi: 10.1128/AAC.34.1.52

Trubiano, J. A., and Padiglione, A. A. (2015). Nosocomial infections in the intensive care unit. *Anaesth. Intensive Care* 16, 598–602. doi: 10.1016/j.mpaic.2015.09.010

Turner, K. H., Everett, J., Trivedi, U., Rumbaugh, K. P., and Whiteley, M. (2014). Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection. *PLoS Genet.* 10:e1004518. doi: 10.1371/journal.pgen.1004518

Ueda, A., and Wood, T. K. (2009). Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog.* 5:e1000483. doi: 10.1371/journal.ppat.1000483

Ueda, A., and Wood, T. K. (2010). Tyrosine phosphatase TpbA of *Pseudomonas aeruginosa* controls extracellular DNA via cyclic diguanylic acid concentrations. *Environ. Microbiol.* 2, 449–455. doi: 10.1111/j.1758-2229.2010.00171.x

Umadevi, S., Joseph, N. M., Kumari, K., Easow, J. M., Kumar, S., Stephen, S., et al. (2011). Detection of extended spectrum beta lactamases, *ampc* beta-lactamases and metallobetalactamases in clinical isolates of ceftazidime resistant *Pseudomonas aeruginosa*. *Braz. J. Microbiol.* 42, 1284–1288. doi: 10.1590/S1517-83822011000400006

Valentini, M., and Filloux, A. (2016). Biofilms and cyclic di-GMP (c-di-GMP) signaling: lessons from *Pseudomonas aeruginosa* and other bacteria. *J. Biol. Chem.* 291, 12547–12555. doi: 10.1074/jbc.R115.711507

van Belkum, A., Soriaga, L. B., LaFave, M. C., Akella, S., Veyrieras, J. B., Barbu, E. M., et al. (2015). Phylogenetic distribution of CRISPR-Cas systems in antibiotic-resistant *Pseudomonas aeruginosa*. *MBio* 6:e01796-15. doi: 10.1128/mBio.01796-15

van Delden, C., Comte, R., and Bally, A. M. (2001). Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. *J. Bacteriol.* 183, 5376–5384. doi: 10.1128/JB.183.18.5376-5384.2001

Van Delden, C., and Iglewski, B. H. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg. Infect. Dis.* 4, 551–560. doi: 10.3201/eid0404.980405

Van Gennip, M., Christensen, L. D., Alhede, M., Phipps, R., Jensen, P. Ø., Christophersen, L., et al. (2009). Inactivation of the *rhlA* gene in *Pseudomonas aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes. *APMIS* 117, 537–546. doi: 10.1111/j.1600-0463.2009.02466.x

Van Melderen, L. (2010). Toxin-antitoxin systems: why so many, what for? *Curr. Opin. Microbiol.* 13, 781–785. doi: 10.1016/j.mib.2010.10.006

Varga, J. J., Barbier, M., Mulet, X., Bielecki, P., Bartell, J. A., Owings, J. P., et al. (2015). Genotypic and phenotypic analyses of a *Pseudomonas aeruginosa* chronic bronchiectasis isolate reveal differences from cystic fibrosis and laboratory strains. *BMC Genomics* 16:883. doi: 10.1186/s12864-015-2069-0

Venter, H., Mowla, R., Ohene-Agyei, T., and Ma, S. (2015). RND-type drug e fflux pumps from Gram-negative bacteria: molecular mechanism and inhibition. *Front. Microbiol.* 6:377. doi: 10.3389/fmicb.2015.00377

Venturi, V. (2006). Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol. Rev.* 30, 274–291. doi: 10.1111/j.1574-6976.2005.00012.x

Viducic, D., Ono, T., Murakami, K., Susilowati, H., Kayama, S., Hirota, K., et al. (2006). Functional analysis of *spoT, relA* and *dksA* genes on quinolone tolerance in *Pseudomonas aeruginosa* under nongrowing condition. *Microbiol. Immunol.* 50, 349–357. doi: 10.1111/j.1348-0421.2006.tb03793.x

Visca, P., Imperi, F., and Lamont, I. L. (2007). Pyoverdine siderophores: from biogenesis to biosignificance. *Trends Microbiol.* 15, 22–30. doi: 10.1016/j.tim.2006.11.004

Vogt, S. L., Green, C., Stevens, K. M., Day, B., Erickson, D. L., Woods, D. E., et al. (2011). The stringent response is essential for *Pseudomonas aeruginosa* virulence in the rat lung agar bead and *Drosophila melanogaster* feeding models of infection. *Infect. Immun.* 79, 4094–4104. doi: 10.1128/IAI.00193-11

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., and Iglewski, B. H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* 185, 2080–2095. doi: 10.1128/JB.185.7.2080-2095.2003

Wang, H., Tu, F., Gui, Z., Lu, X., and Chu, W. (2013). Antibiotic resistance profiles and quorum sensing-dependent virulence factors in clinical isolates of *Pseudomonas aeruginosa*. *Indian J. Microbiol.* 53, 163–167. doi: 10.1007/s12088-013-0370-7

Wang, S., Parsek, M. R., Wozniak, D. J., and Ma, L. Z. (2013). A spider web strategy of type IV pili-mediated migration to build a fibre-like Psl polysaccharide matrix in *Pseudomonas aeruginosa* biofilms. *Environ. Microbiol.* 15, 2238–2253. doi: 10.1111/1462-2920.12095

Wang, X., and Wood, T. K. (2011). Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl. Environ. Microbiol.* 77, 5577–5583. doi: 10.1128/AEM.05068-11

Wang, X. X., Lord, D. M., Cheng, H. Y., Osbourne, D. O., Hong, S. H., Sanchez-Torres, V., et al. (2012). A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nat. Chem. Biol.* 8, 855–861. doi: 10.1038/nchembio.1062

Wang, Y., Hay, I. D., Rehman, Z. U., and Rehm, B. H. (2015). Membrane-anchored MucR mediates nitrate-dependent regulation of alginate production in *Pseudomonas aeruginosa*. *Appl. Microbiol. Biotechnol.* 99, 7253–7265. doi: 10.1007/s00253-015-6591-4

Wei, Q., Tarighi, S., Dotsch, A., Haussler, S., Müsken, M., Wright, V. J., et al. (2011). Phenotypic and genome-wide analysis of an antibiotic-resistant small colony variant (SCV) of *Pseudomonas aeruginosa*. *PLoS ONE* 6:e29276. doi: 10.1371/journal.pone.0029276

Wen, Y. R., Behiels, E., and Devreese, B. (2014). Toxin- Antitoxin systems: their role in persistence, biofilm formation, and pathogenicity. *Pathog. Dis.* 70, 240–249. doi: 10.1111/2049-632X.12145

Whiteley, M., Lee, K. M., and Greenberg, E. P. (1999). Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13904–13909. doi: 10.1073/pnas.96.24.13904

Williams, J. J., Halvorsen, E. M., Dwyer, E. M., DiFazio, R. M., and Hergenrother, P. J. (2011). Toxin-antitoxin (TA) systems are prevalent and transcribed in clinical isolates of *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 322, 41–50. doi: 10.1111/j.1574-6968.2011.02330.x

Williams, P., and Camara, M. (2009). Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr. Opin. Microbiol.* 12, 182–191. doi: 10.1016/j.mib.2009.01.005

Winstanley, C., O'Brien, S., and Brockhurst, M. A. (2016). *Pseudomonas aeruginosa* evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. *Trends Microbiol.* 24, 327–337. doi: 10.1016/j.tim.2016.01.008

Wittgens, A., Tiso, T., Arndt, T. T., Wenk, P., Hemmerich, J., Müller, C., et al. (2011). Growth independent rhamnolipid production from glucose using the non-pathogenic *Pseudomonas putida* KT2440. *Microb. Cell Fact.* 10:80. doi: 10.1186/1475-2859-10-80

Wong, A., Rodrigue, N., and Kassen, R. (2012). Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. *PLoS Genet.* 8:e1002928. doi: 10.1371/journal.pgen.1002928

Wood, T. L., and Wood, T. K. (2016). The HigB/HigA toxin/antitoxin system of *Pseudomonas aeruginosa* influences the virulence factors pyochelin, pyocyanin, and biofilm formation. *Microbiologyopen* 5, 499–511. doi: 10.1002/mbo3.346

Workentine, M. L., Sibley, C. D., Glezerson, B., Purighalla, S., Norgaard-Gron, J. C., Parkins, M. D., et al. (2013). Phenotypic heterogeneity of *Pseudomonas aeruginosa* populations in a cystic fibrosis patient. *PLoS ONE* 8:e60225. doi: 10.1371/journal.pone.0060225

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C., et al. (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J. Clin. Invest.* 109, 317–325. doi: 10.1172/JCI0213870

van 't Wout, E. F. A., van Schadewijk, A., van Boxtel, R., Dalton, L. E., Clarke, H. J., Tommassen, J., et al. (2015). Virulence factors of *Pseudomonas aeruginosa* induce both the unfolded protein and integrated stress responses in airway epithelial cells. *PLoS Pathog.* 11:e1004946. doi: 10.1371/journal.ppat.1004946

Wu, J., Long, Q., and Xie, J. (2010). (p)ppGpp and drug resistance. *J. Cell. Physiol.* 224, 300–304. doi: 10.1002/jcp.22158

Wu, L., Estrada, O., Zaborina, O., Bains, M., Shen, L., Kohler, J. E., et al. (2005). Recognition of host immune activation by *Pseudomonas aeruginosa*. *Science* 309, 774–777. doi: 10.1126/science.1112422

Xiong, Y. Q., Caillon, J., Drugeon, H., Potel, G., and Baron, D. (1996). Influence of pH on adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides and their postantibiotic effects. *Antimicrob. Agents Chemother.* 40, 35–39.

Ye, L., Cornelis, P., Guillemyn, K., Ballet, S., and Hammerich, O. (2014). Structure revision of N-mercapto-4-formylcarbostyril produced by *Pseudomonas fluorescens* G308 to 2-(2-hydroxyphenyl)thiazole-4-carbaldehyde [aeruginaldehyde]. *Nat. Prod. Commun.* 9, 789–794.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., et al. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev. Cell* 3, 593–603. doi: 10.1016/S1534-5807(02)00295-2

Zaborin, A., Romanowski, K., Gerdes, S., Holbrook, C., Lepine, F., Long, J., et al. (2009). Red death in *Caenorhabditis elegans* caused by *Pseudomonas aeruginosa* PAO1. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6327–6332. doi: 10.1073/pnas.0813199106

Zegans, M. E., Wagner, J. C., Cady, K. C., Murphy, D. M., Hammond, J. H., and O'Toole, G. A. (2009). Interaction between bacteriophage DMS3 and host CRISPR region inhibits group behaviors of *Pseudomonas aeruginosa*. *J. Bacteriol.* 191, 210–219. doi: 10.1128/JB.00797-08

Zegans, M. E., Wozniak, D., Griffin, E., Toutain-Kidd, C. M., Hammond, J. H., Garfoot, A., et al. (2012). *Pseudomonas aeruginosa* exopolysaccharide Psl promotes resistance to the biofilm inhibitor polysorbate 80. *Antimicrob. Agents Chemother.* 56, 4112–4122. doi: 10.1128/AAC.00373-12

Zhang, L., Li, X. Z., and Poole, K. (2001). Fluoroquinolone susceptibilities of efflux-mediated multidrug-resistant *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia*. *J. Antimicrob. Chemother.* 48, 549–552. doi: 10.1093/jac/48.4.549

Zhao, J., Jiang, H., Cheng, W., Wu, J., Wang, J., and Dong, L. (2015). The role of quorum sensing system in antimicrobial induced *ampC* expression in *Pseudomonas aeruginosa* biofilm. *J. Basic Microbiol.* 55, 671–678. doi: 10.1002/jobm.201300987

Zhao, K., Tseng, B. S., Beckerman, B., Jin, F., Gibiansky, M. L., Harrison, J. J., et al. (2013). Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. *Nature* 497, 388–391. doi: 10.1038/nature12155

Zhu, K., and Rock, C. O. (2008). RhlA converts β-hydroxyacyl-acyl carrier protein intermediates in fatty acid synthesis to the β-hydroxydecanoyl-β-hydroxydecanoate component of rhamnolipids in *Pseudomonas aeruginosa*. *J. Bacteriol.* 190, 3147–3154. doi: 10.1128/JB.00080-08

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

REVIEW
published: 03 April 2017
doi: 10.3389/fcimb.2017.00106

In vivo and *In vitro* Interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp.

*An Hotterbeekx[1], Samir Kumar-Singh[1,2], Herman Goossens[1] and Surbhi Malhotra-Kumar[1]**

[1] *Laboratory of Medical Microbiology, Vaccine and Infectious Disease Institute, University of Antwerp, Wilrijk, Belgium,*
[2] *Molecular Pathology Group, Cell Biology and Histology, University of Antwerp, Wilrijk, Belgium*

The significance of polymicrobial infections is increasingly being recognized especially in a biofilm context wherein multiple bacterial species—including both potential pathogens and members of the commensal flora—communicate, cooperate, and compete with each other. Two important bacterial pathogens that have developed a complex network of evasion, counter-inhibition, and subjugation in their battle for space and nutrients are *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Their strain- and environment-specific interactions, for instance in the cystic fibrosis lung or in wound infections, show severe competition that is generally linked to worse patient outcomes. For instance, the extracellular factors secreted by *P. aeruginosa* have been shown to subjugate *S. aureus* to persist as small colony variants (SCVs). On the other hand, data also exist where *S. aureus* inhibits biofilm formation by *P. aeruginosa* but also protects the pathogen by inhibiting its phagocytosis. Interestingly, such interspecies interactions differ between the planktonic and biofilm phenotype, with the extracellular matrix components of the latter likely being a key, and largely underexplored, influence. This review attempts to understand the complex relationship between *P. aeruginosa* and *Staphylococcus* spp., focusing on *S. aureus*, that not only is interesting from the bacterial evolution point of view, but also has important consequences for our understanding of the disease pathogenesis for better patient management.

Keywords: microbial interactions, *S. aureus*, quorum-sensing, cystic fibrosis, biofilm

OPEN ACCESS

Edited by:
Ghassan M. Matar,
American University of Beirut, Lebanon

Reviewed by:
Robert J. C. McLean,
Texas State University, USA
Sarah Maddocks,
Cardiff Metropolitan University, UK

****Correspondence:***
Surbhi Malhotra-Kumar
surbhi.malhotra@uantwerpen.be

Received: *30 January 2017*
Accepted: *16 March 2017*
Published: *03 April 2017*

Citation:
Hotterbeekx A, Kumar-Singh S, Goossens H and Malhotra-Kumar S (2017) In vivo and In vitro Interactions between Pseudomonas aeruginosa and Staphylococcus spp. Front. Cell. Infect. Microbiol. 7:106. doi: 10.3389/fcimb.2017.00106

INTRODUCTION

Over the past decade there is a growing appreciation that the biofilm mode of growth is the most common lifestyle adopted by bacteria (Hall-Stoodley et al., 2004; Burmolle et al., 2014). Biofilms can be defined as surface-associated, structured bacterial communities embedded in an extracellular matrix (Hall-Stoodley et al., 2004). Living in a biofilm provides protection in a stressful environment where mechanical stress, desiccation, and biocides are common threats (Donlan and Costerton, 2002; Flemming and Wingender, 2010). Multiple species frequently exist together in a single biofilm, where they either improve the fitness of one another or compete for space and nutrients (Jefferson, 2004; Billings et al., 2013; Burmolle et al., 2014; DeLeon et al., 2014). Most bacteria have developed interaction strategies to communicate within and between species in a cell density-dependent manner, for example, by using small diffusible molecules in a process called quorum-sensing (Federle and Bassler, 2003; Li and Tian, 2012). Furthermore, many bacteria excrete

antimicrobial components, also often regulated by quorum-sensing, to eliminate competitors (Federle and Bassler, 2003; Li and Tian, 2012). Indeed, these multispecies interactions within the biofilm are important for the inhabiting bacteria and, given the increasing evidence of the link between biofilm-associated pathogens and disease, also from a clinical point of view (Donlan and Costerton, 2002; Li and Tian, 2012).

S. aureus and *P. aeruginosa* are important pathogens causing a wide variety of infections, including pneumonia in cystic fibrosis (CF) patients, healthcare associated pneumonia and chronic wounds (Harrison, 2007; Fazli et al., 2009; Cystic Fibrosis Foundation Patient Registry, 2012). Initially, only an antagonistic relationship between both organisms was described as the presence of one is associated with the absence of the other in CF and both are rarely found in close association in chronic wounds. *S. aureus* mostly resides on the wound surface whereas *P. aeruginosa* is found in the deep layers (Kirketerp-Moller et al., 2008; Fazli et al., 2009). We also recently showed a negative correlation between presence of *P. aeruginosa* and the total species diversity in *in vivo* endotracheal tube biofilms and a low co-occurrence of *P. aeruginosa* with *Staphylococcus epidermidis* (Hotterbeekx et al., 2016). Nonetheless, recent studies have also co-isolated *P. aeruginosa* and Gram-positive bacteria, including *S. aureus*, from the same infection site where increased virulence and/or antibiotic resistance is described (Duan et al., 2003; Kirketerp-Moller et al., 2008; Fazli et al., 2009; Dalton et al., 2011; Korgaonkar et al., 2013). After describing first *in vivo* observations occurring in human diseases, we will discuss and summarize *in vitro* data from the current literature on potential mechanisms of interactions between *P. aeruginosa* and *Staphylococcus* spp., primarily *S. aureus*.

CO-OCCURRENCE OF *P. AERUGINOSA* AND *S. AUREUS IN VIVO* IS LINKED TO WORSE DISEASE OUTCOMES

CF is a typical example of a biofilm-related infection wherein *P. aeruginosa* and *S. aureus* are frequently isolated from the lungs of these patients (Harrison, 2007; Hauser et al., 2011; Cystic Fibrosis Foundation, 2014). While, *S. aureus* is mostly acquired during childhood, the presence of *P. aeruginosa* is associated with increasing age and worsening patient prognosis (Sagel et al., 2009; Hauser et al., 2011; Cystic Fibrosis Foundation, 2014). An increasing incidence of *P. aeruginosa* with age has been shown to coincide with a decreasing *S. aureus* incidence in CF patients (Harrison, 2007; Cystic Fibrosis Foundation, 2014), data that primarily indicates an antagonistic relationship between the two pathogens. However, in cases where *P. aeruginosa* and *S. aureus* have been co-isolated, both pathogens seem to contribute independently and additively to the disease severity (Sagel et al., 2009; Hauser et al., 2011), presenting as increased lung inflammation and consequently increased lung damage compared to infection with a single pathogen (Sagel et al., 2009). Furthermore, due to repeated antibiotic therapy, CF patients also carry higher levels of methicillin-resistant *S. aureus* (MRSA) that is associated with a worse lung function compared to methicillin- sensitive *S. aureus* (MSSA) but only in combination with *P. aeruginosa* (Hubert et al., 2013).

Chronic wounds are another example of biofilm-related infections wherein co-presence of *P. aeruginosa* and *S. aureus* has been shown to result in delayed wound healing compared to single species infections (Dalton et al., 2011; Seth et al., 2012; Pastar et al., 2013). In a pig wound model, infections initiated by *in vitro* preformed dual species biofilm caused a significant suppression of keratinocyte growth factor 1 (KGF1), which is responsible for re-epithelialization and wound closure (Pastar et al., 2013). In a rabbit ear-wound model, mixed species infection of *S. aureus* and *P. aeruginosa* caused an increased expression of the pro-inflammatory cytokines IL-1β and TNF-α, indicating a higher inflammatory response compared to single species infection (Seth et al., 2012). Moreover, *S. aureus* and *P. aeruginosa* reached an equilibrium after 12 days of infection, with *P. aeruginosa* being the dominant pathogen (Seth et al., 2012). In a mouse chronic wound model infected with *in vitro* preformed four-species biofilm and monitored up to 12 days, presence of multiple species was found to significantly delay wound healing only at 8 days post-infection (Dalton et al., 2011). However, polymicrobial infections showed increased antimicrobial tolerance compared to single species infection with *P. aeruginosa* in this study (Dalton et al., 2011). These studies suggest that, despite the constraints of different host backgrounds, multispecies infections can lead to delayed wound healing, increased inflammation and increased antibiotic tolerance, which all add to a worse patient outcome. *P. aeruginosa* is often the dominant pathogen due to its wide array of mechanisms to adapt to changing hostile environments, which allows colonization in a variety of niches. When *P. aeruginosa* encounters other bacteria like *S. aureus*, it can co-exist or take over the biofilm through production of various quorum-sensing regulated factors. Section Extracellular products of *Staphylococcus* spp. impact *P. aeruginosa* virulence *in vivo* discusses *in vivo* animal studies exploring production of *P. aeruginosa* virulence factors in the presence of *S. aureus*.

EXTRACELLULAR PRODUCTS OF *STAPHYLOCOCCUS* SPP. IMPACT *P. AERUGINOSA* VIRULENCE *IN VIVO*

P. aeruginosa possesses a wide range of extracellular factors to survive and invade human tissues, often by modulating the immune system. The complex interplay between biofilms and the host immune response are reviewed in detail by Watters et al. (2016). Here, we discuss four molecules in particular which are upregulated in the presence of Gram-positive bacteria: LasB elastase, rhamnolipids, exotoxins, and phenazines (**Figure 1**). LasB elastase is an extracellular protease capable of digesting the lung surfactant, the pulmonary antimicrobial enzyme lysozyme, and transferrin, as well as slowing down the ciliary movement (Hauser, 2009). In addition, LasB impairs uptake of *P. aeruginosa* by macrophages and its protease activity leads to lung tissue

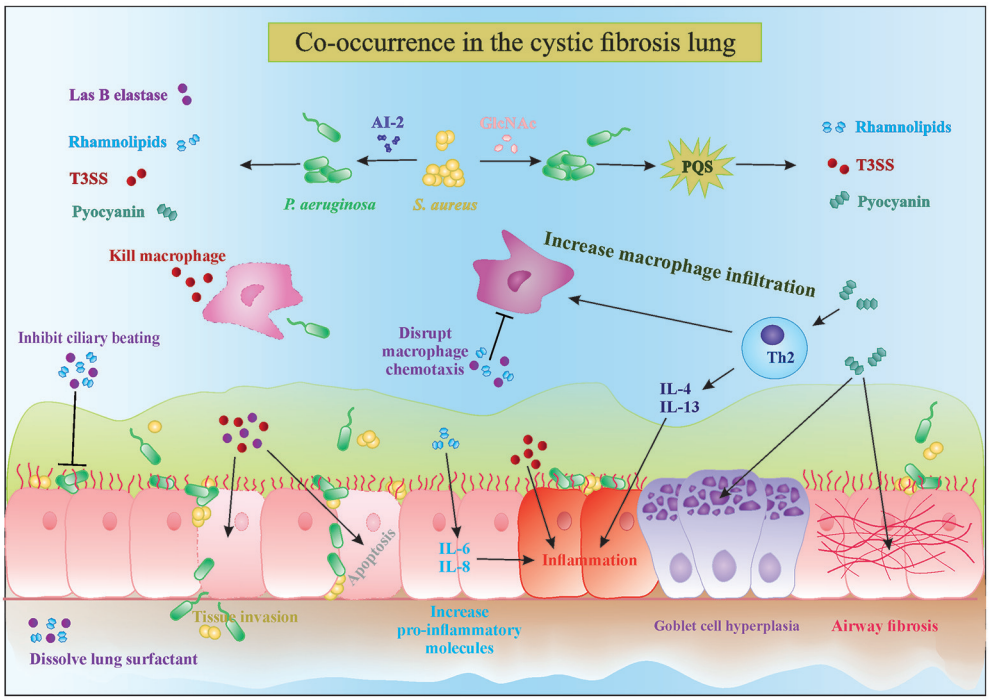


FIGURE 1 | Interactions in the cystic fibrosis lung. The complex interplay between microbial competition and the human immune system results in increased secretion of pro-inflammatory cytokines, microbial virulence factors and consequently tissue damage and bacterial invasion of the epithelial barrier. Mixed species infections lead to a stronger decrease in ciliary beating and increased goblet cell hyperplasia and tissue fibrosis, which are characteristic of cystic fibrosis disease progression. PQS, Pseudomonas quinolone signal; T3SS, type 3 secretion system; AI-2, autoinducer 2.

damage, thereby decreasing pulmonary function and facilitating dissemination into the bloodstream (Strateva and Mitov, 2011). Similar to LasB, rhamnolipids are glycolipidic biosurfactants that interfere with the lung surfactant activity by solubilizing the phospholipids and with airway immune response by disrupting the polymorphonuclear leucocyte chemotaxis and macrophage function, and also inhibit ciliary beating (Soberon-Chavez et al., 2005; Jensen et al., 2007). Furthermore, rhamnolipids increase inflammation by stimulating the release of the pro-inflammatory cytokines IL-6 and IL-8 by the airway epithelium (Soberon-Chavez et al., 2005). An excessive inflammatory response and associated tissue damage is also induced by the release of exotoxins ExoT, ExoS, and ExoY by the type III secretion system. The type III secretion system is a needle-like structure directly injecting exotoxins into other bacteria, macrophages and epithelial cells, thereby killing them (Hauser, 2009). The fourth type of molecules, phenazines, are pigments produced by a large number of *Pseudomonas* spp., and have been shown to be involved in mediating microbial interactions as well as in CF disease progression. Pyocyanin is the most important phenazine and its production in the CF lung was shown to lead to goblet cell hyperplasia, airway fibrosis and alveolar airspace destruction (Caldwell et al., 2009; Strateva and Mitov, 2011). Pyocyanin causes an imbalance between the T helper type 1 (Th1) and type 2 (Th2) cytokines, leading to overproduction of Th2 cytokines IL-4 and IL-13 and increased macrophage infiltration (Caldwell et al., 2009). Essentially, release of these extracellular molecules by *P. aeruginosa*, partly in response to the presence of *S. aureus*, leads to increased tissue damage due to their cytotoxic and immune-modulatory effects, which also helps *P. aeruginosa* survival. Interestingly, not only *S. aureus* but Gram-positive commensals (coagulase negative staphylococci and viridans streptococci) can also alter the virulence of *P. aeruginosa* in a similar fashion (Duan et al., 2003). Such immune modulation and evasion by collective bacterial species might underlie the worsened patient prognosis observed in multispecies infections (**Figure 1**). Sections AI-2 in the CF Lung Increases *P. aeruginosa* Virulence and Might Be an Important Therapeutic Target and N-Acetyl Glucosamine Sensing Enhances the Production of *P. aeruginosa* Extracellular Virulence Factors discuss the known mechanisms of how extracellular products of staphylococci modulate the four major virulence factors *P. aeruginosa* that were discussed above.

AI-2 in the CF Lung Increases *P. aeruginosa* Virulence and Might Be an Important Therapeutic Target

Autoinducer-2 (AI-2) is a small diffusible quorum-sensing molecule produced by several bacteria, including staphylococci, and has been shown to cause upregulation of several major virulence genes of *P. aeruginosa* discussed above, including extracellular protease (*lasB*), rhamnosyltransferase involved in rhamnolipid synthesis (*rhlA*), exotoxins (*exoT, exoS, exoY*)

and phenazines (*phzA1* and *phzA2*; Li et al., 2015). Induction of *P. aeruginosa* virulence by AI-2 was shown both *in vitro* after screening of a random *lux* reporter-based promotor library and *in vivo* in rat lung infection and *Drosophila* chronic infection models (Duan et al., 2003; Sibley et al., 2008; Li et al., 2015). AI-2 mediated quorum-sensing is now recognized as a universal language of interspecies communication regulating a wide variety of genes involved in virulence and biofilm formation in a cell-density dependent manner in a number of micro-organisms, including non-producers like *P. aeruginosa* (Rezzonico et al., 2012). Furthermore, AI-2 has been detected in substantial amounts in the sputum of CF patients and in infected rats (Duan et al., 2003), raising the possibility of interruption of AI-2 signaling to either slow down disease progression or hasten the healing process.

A promising approach is the use of AI-2 analogs like D-ribose that block the AI-2 pathway and inhibit *P. aeruginosa* virulence. Wang et al showed in a rat model of mechanical ventilation that co-inoculation of *P. aeruginosa* and *Streptococcus mitis* resulted in increased biomass, lung damage, and rat mortality compared to infection with only *P. aeruginosa* (Wang et al., 2016). Treatment with D-ribose of both the single and dual species infections showed a significant decrease in biomass and lowering of rat mortality in the latter group due to interference with AI-2 signaling (Wang et al., 2016). Inhibition of *P. aeruginosa* virulence is not only beneficial because the *P. aeruginosa*-mediated damage is reduced but also because the immune system is less stimulated (**Figure 1**). Further studies showing the benefits of non-toxic biofilm inhibitors such as D-ribose in patient populations are awaited.

N-Acetyl Glucosamine Sensing Enhances the Production of *P. aeruginosa* Extracellular Virulence Factors

Another molecule that increases the virulence of *P. aeruginosa* and is commonly found in the CF lung is N-acetyl glucosamine (GlcNAc). GlcNAc is part of the Gram-positive cell wall polymer peptidoglycan and induces the virulence of *P. aeruginosa* by enhancing the *Pseudomonas* quinolone signal (PQS), which controls the production of extracellular virulence factors like pyocyanin, elastase, rhamnolipids and HQNO (discussed in Section Pseudomonas Quinolone Signal Regulates the Production of Anti-Staphylococcal 4-Hydroxy-2-Heptylquinoline N-Oxide (HQNO); Deziel et al., 2004; Williams and Camara, 2009; Jimenez et al., 2012; Korgaonkar et al., 2013; **Figures 1**, **2**). The PQS, with 2-heptyl-3-hydroxy-4-quilonone as the main effector molecule, is one of the three quorum-sensing systems present in *P. aeruginosa*. PQS is positively regulated by LasR and negatively regulated by RhlR, the two other quorum-sensing systems of *P. aeruginosa* with N-acylhomoserine lactone as main effector molecule (Jimenez et al., 2012). *P. aeruginosa* has the ability to sense the peptidoglycan shed by the Gram positive commensal flora and in response increase the production of antimicrobials. The enhanced virulence in the presence of GlcNAc from Gram-positive bacteria was demonstrated *in vivo* in a *Drosophila* and *Galleria mellonella* infection model (Korgaonkar et al., 2013; Whiley et al., 2014). Both GlcNAc and AI-2 sensing are examples where *P. aeruginosa* can sense its environment and generate the appropriate response to eliminate competitors by producing several virulence factors, which also has a negative impact on the host.

P. AERUGINOSA PRODUCES A WIDE VARIETY OF MOLECULES THAT INHIBIT *S. AUREUS IN VITRO*

P. aeruginosa produces many molecules to compete with other microorganisms for space and nutrients. The number of molecules, quantities produced and even the structure of these molecules vary between different strains of *P. aeruginosa* as well as between different growth conditions (planktonic vs. biofilm; the presence of host factors, antibiotics etc.). In order to achieve a better understanding of complex interplay of the different compounds in *in vivo* biofilm-related infections, it is necessary to dissect this complex system into individual subsystems and investigate each compound individually. In the following sections, we discuss the effect of different molecules produced by *P. aeruginosa* on *S. aureus in vitro*. An overview of the extracellular molecules produced by *P. aeruginosa* and their effect on *S. aureus* is shown in **Figure 2**.

Pseudomonas Quinolone Signal Regulates the Production of Anti-Staphylococcal 4-Hydroxy-2-Heptylquinoline N-Oxide (HQNO)

P. aeruginosa strongly reduces or completely outcompetes *S. aureus* during co-culture in many *in vitro* model systems, both planktonic and biofilm (Palmer et al., 2005; Baldan et al., 2014; DeLeon et al., 2014). This anti-staphylococcal activity of *P. aeruginosa* was first described by Lightbown and Jackson (1956), who identified 4-hydroxy-2-heptylquinoline N-oxide (HQNO) as a major compound produced by *P. aeruginosa* that inhibited the cytochrome systems of some bacteria, including *S. aureus* (Lightbown and Jackson, 1956). The same phenomenon was again described by Machan et al. in 1991 by testing the culture supernatant of fifty *P. aeruginosa* clinical isolates on 261 staphylococci (Machan et al., 1991). The growth of all staphylococci was reduced by each of the *P. aeruginosa* strains, although the extent of inhibition was strain-dependent. The factor responsible for this phenomenon was again identified as HQNO (Machan et al., 1992). HQNO is the major compound produced by the *pqsABCDE* operon, which is regulated by the quorum-sensing system PQS. Although HQNO is described as an antistaphylococcal compound, it has no lytic activity against *S. aureus* itself but rather slows down the growth by inhibiting oxidative respiration (**Figure 2**, right upper panel; Williams and Camara, 2009). Exposure to an HQNO source suppresses the growth of *S. aureus,* resulting in small colonies

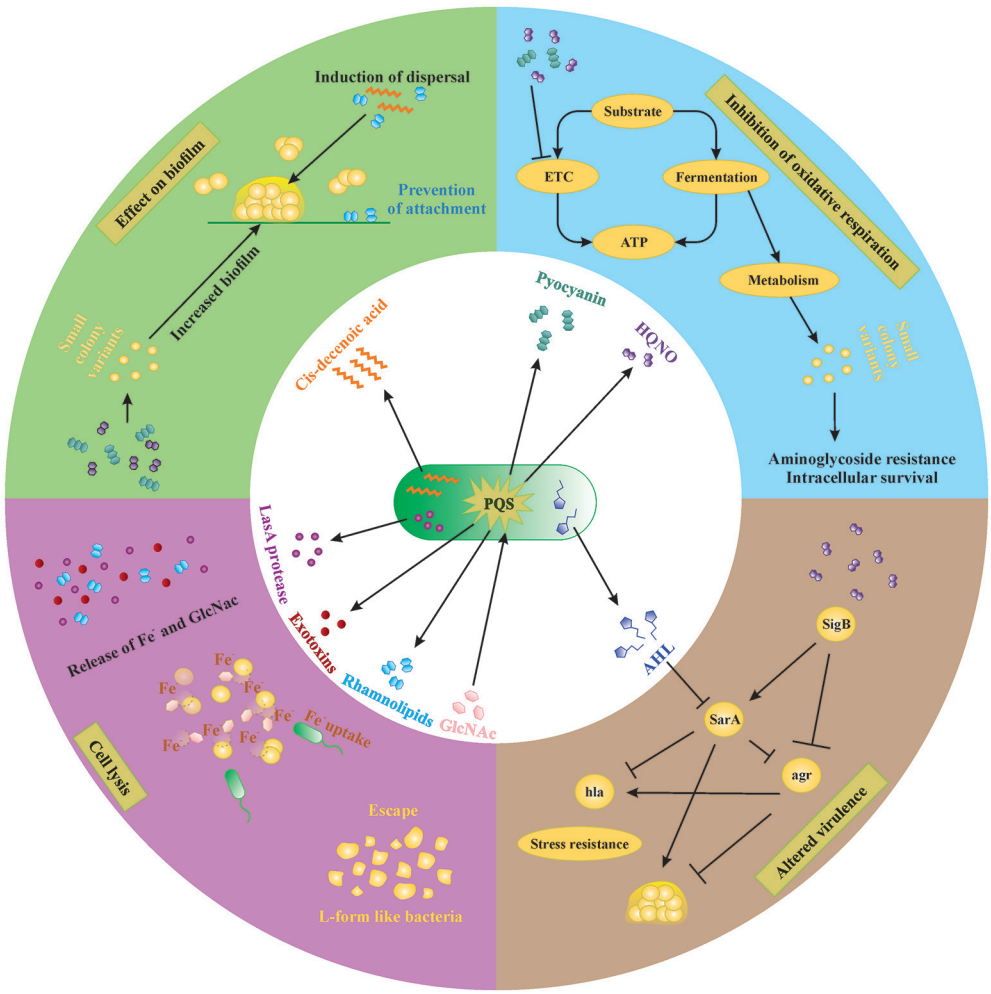


FIGURE 2 | Small molecules secreted by *P. aeruginosa* and *S. aureus*. Extracellular factors produced by *P. aeruginosa* affect biofilm formation, oxidative respiration, cell lysis and virulence of *S. aureus*. Lysis of *S. aureus* leads to increased extracellular iron and N-acetyl glucosamine (GlcNac), which are sensed by *P. aeruginosa*. AHL, N-acyl homoserine lactone; HQNO, 4-hydroxy-2-heptylquinoline N-oxide; PQS, Pseudomonas quinolone signal; GlcNac, N-acetyl glucosamine.

which are easily missed in diagnostic cultures (Hoffman et al., 2006). These so-called SCVs represent a different phenotype with specific characteristics and will be discussed later in this review. Furthermore, HQNO can be detected at active concentrations in the sputum of CF patients infected with *P. aeruginosa*, suggesting that HQNO has the same effect in the lungs of CF patients as it has *in vitro* (Hoffman et al., 2006). Although HQNO is one of the most important and well-studied antistaphylococcal compounds, it is not the only factor slowing down the growth and inhibiting oxidative respiration in staphylococci.

Pyocyanin Inhibits Oxidative Respiration in *S. aureus*

Pyocyanin is one of the numerous pigmented phenazines produced by *P. aeruginosa* and an important virulence factor. Pyocyanin is produced during *Pseudomonas* biofilm formation, has a role in acute and chronic airway infections, enables anaerobic survival and serves as a redox-active antimicrobial compound (Biswas et al., 2009; Caldwell et al., 2009). Furthermore, by its inter-and intracellular signaling, pyocyanin enables *P. aeruginosa* to successfully compete with other bacteria and even fungi (Gibson et al., 2009; Toyofuku et al., 2010; Tashiro et al., 2013). Like HQNO, pyocyanin also blocks the oxidative respiration and inhibits growth of *S. aureus,* also selecting for the SCV phenotype (Biswas et al., 2009; **Figure 2**, right upper panel). The production of pyocyanin can be observed after 8 h of culture, around the same time when a strong reduction of *S. aureus* cells occurs during co-culture (Biswas et al., 2009; Tashiro et al., 2013). Furthermore, the presence of Gram-positive organisms, including some *Staphylococcus* spp., can induce pyocyanin production in *P. aeruginosa* by stimulating the PQS system (Korgaonkar and Whiteley, 2011; Whiley et al., 2014; **Figure 2**, middle panel). In addition, exposure to pyocyanin in the airways leads to pulmonary damage and contributes to CF pathogenesis (Caldwell et al., 2009).

Therefore, pyocyanin seems to be an antagonistic compound secreted to provide a competitive advantage to *P. aeruginosa* by harming *S. aureus,* other Gram-positive bacteria as well as the host.

LasA Protease or Staphylolysin Effectively Lyses *S. aureus* Cells

P. aeruginosa secretes a staphylolytic endopeptidase called LasA protease or staphylolysin, which degrading pentaglycine in the cell wall of *S. aureus* causing cell lysis (Kessler et al., 1993). *P. aeruginosa* might use LasA protease to compete with staphylococci but also to acquire iron from *S. aureus* (Mashburn et al., 2005; **Figure 2**, left lower panel). Because freely available iron is often limited, *P. aeruginosa* has developed several strategies to scavenge iron, like the synthesis of iron chelating siderophores, pyoverdin, and pyochelin (Diggle et al., 2007). Transcription patterns of iron-regulated genes of *P. aeruginosa* in the presence of *S. aureus in vivo* are the same as in high-iron conditions *in vitro*, suggesting that *S. aureus* might be an iron source for *P. aeruginosa* (Mashburn et al., 2005). This type of interaction is, however, only important when both species are located close together, like in multi-species biofilms (Mashburn et al., 2005). The specificity of the LasA protease for staphylococci makes it a potential therapeutic candidate against staphylococcal infections especially those cause by antibiotic resistant MRSA strains as shown in a rat model of endophthalmitis (Barequet et al., 2009). However, *S. aureus* can survive LasA by the emergence of L-form-like colonies, which lack a cell wall (Falcon et al., 1989), although the role of L-form like colonies in disease remains rather vague.

Cis-2-Decenoic Acid Induces Biofilm Dispersal in a Broad Range of Organisms Including *P. aeruginosa*

Interspecies competition in biofilms not only occurs by inhibiting or killing the other species but also by inducing its dispersal. Biofilm dispersal is mainly induced when the environment becomes less favorable, like in case of nutrient depletion, and is extensively reviewed by Petrova and Sauer (2016). The exact mechanisms that induce biofilm dispersal are currently unknown, although several factors have been investigated (Hall-Stoodley and Stoodley, 2005; Davies and Marques, 2009). Since most bacteria reside in a biofilm consisting of multiple species *in vivo*, the dispersal signal must be recognized by a wide range of species (Davies and Marques, 2009). One class of such molecules are the *cis*-monosaturated fatty acids, which are small extracellular messenger molecules with broad inter-phylum and even inter-kingdom activities (Davies and Marques, 2009). *P. aeruginosa* produces *cis*-2-decenoic acid, which induces a dispersion response in biofilms formed by a range of Gram-negative and Gram-positive bacteria, including *S. aureus* (**Figure 2**, left upper panel), yeast as well as in *P. aeruginosa* (Davies and Marques, 2009). Interestingly, Davies et al showed that dispersion was only induced when the microcolonies reached a minimum of 40 μm diameter and 10 μm of thickness, indicating that a certain threshold concentration is needed for *cis*-2-decenoic acid to become active (Davies and Marques, 2009). This molecule could possibly be employed to disrupt biofilms on surfaces, followed by disinfectants that can successfully clear planktonic bacteria.

Rhamnolipids Promote Biofilm Dispersal and Inhibit Adhesion

Most *P. aeruginosa* strains produce rhamnolipids, biosurfactants consisting of one or two rhamnose molecules linked to one or two fatty acids (Soberon-Chavez et al., 2005). While *cis*-2-decenoic acid is mainly used as a common signal for dispersion at the final biofilm stages, rhamnolipids are used to dislodge competing bacteria from the biofilm. Many different rhamnolipid homologs are produced, depending on the *Pseudomonas* strain and carbon source, and their synthesis is quorum-sensing regulated (Soberon-Chavez et al., 2005). Rhamnolipids were shown to reduce the surface tension and to have an anti-adhesive and antimicrobial effect on many micro-organisms (Haba et al., 2003; Rodrigues et al., 2006; Zezzi do Valle Gomes and Nitschke, 2012). The amphiphilic nature of rhamnolipids enables them to intercalate into the cell membranes of different microorganisms and form complexes, thereby permeabilizing the membranes and causing leakage of intracellular material (Sotirova et al., 2008). Gram-positive organisms seem more susceptible to rhamnolipid permeabilization than Gram-negative because the presence of lipopolysaccharides protects the cell membranes of the latter against the effect of surfactants (Soberon-Chavez et al., 2005). Furthermore, rhamnolipids were shown to promote biofilm dispersal in many different microorganisms, including *P. aeruginosa* itself, although this effect is strain dependent (Rodrigues et al., 2006). For *S. aureus* and *S. epidermidis*, rhamnolipids were shown to induce biofilm dispersal and to inhibit adhesion in a dose-dependent manner (Rodrigues et al., 2006; Zezzi do Valle Gomes and Nitschke, 2012; Pihl et al., 2013; **Figure 2**, left upper panel).

Long-Chain AHLs Reduce Growth and Virulence of *S. aureus*

The N-acylhomoserine lactone (AHL) system is the most important and most extensively studied quorum-sensing system in *P. aeruginosa*. Many diverse AHLs are produced by various Gram-negative bacteria, all consisting of a homoserine lactone ring that is N-acylated with a fatty acyl group (Jimenez et al., 2012). The length of the acyl chains may vary from 4 to 18 carbons and *P. aeruginosa* mainly produces a short-chain C4-HSL and a long-chain 3-oxo-C12-HSL, although other lengths might also occur (Jimenez et al., 2012). The production of many virulence factors is regulated by AHL, including that of pyocyanin and rhamnolipids (Jimenez et al., 2012). Although it is currently not described that Gram-positive bacteria produce AHLs, they might still be influenced by them (Qazi et al., 2006). For example, growth of *S. aureus* is inhibited by several long chain 3-oxo-AHLs (including C8, C10, C12, and C14 chains) in a concentration dependent manner, with C12 and C14

being the most effective (Qazi et al., 2006). At concentrations below growth inhibition, the function of staphylococcal accessory regulator *sarA* and accessory gene regulator *agr* are strongly reduced (see Section SCV Induction by *P. aeruginosa* is Sigma B Dependent), and consequently their dependent virulence factors like hemolysins, TSST-1, protein A, and fibronectin-binding proteins (**Figure 2**, right lower panel; Qazi et al., 2006). Moreover, inhibition of *agr* might lead to more biofilm formation in *S. aureus* due to reduced detachment, although this study only tested planktonic conditions and requires further research (Qazi et al., 2006). The short chain AHL produced by *P. aeruginosa* seems to have no effect on growth and *agr* expression of *S. aureus* (Qazi et al., 2006). Furthermore, long-chain AHLs produced by other Gram-negative bacteria are likely to have similar effects in *S. aureus,* although, again, more studies are required here.

P. aeruginosa Might also Cause Increased Expression of *S. aureus* Virulence Factors

As described before, wounds infected with both *S. aureus* and *P. aeruginosa* generally show delayed closure compared to the single species infected wounds (Dalton et al., 2011; Seth et al., 2012). In addition to host related factors, one of the reasons for this phenomenon might be the upregulation of *S. aureus* virulence factors during co-infection, as was demonstrated for the MRSA strain USA300 (Pastar et al., 2013). Interestingly, although the growth of USA300 was strongly inhibited by *P. aeruginosa in vitro*, this effect was much weaker *in vivo* (Pastar et al., 2013). Furthermore, co-infection of wounds in a pig model induced *S. aureus* virulence factors *hla* and *pvl*, encoding α-hemolysin and Panton-Valentine leucocidin (**Figure 2**, right lower panel; Pastar et al., 2013). Another example of increased virulence of *S. aureus* in the presence of *P. aeruginosa* is the induction of staphyloxanthin production observed in a white *S. aureus variant* isolated from a soft tissue wound (Antonic et al., 2013). This strain possessed an intact and functional *crtOPQMN* operon, which is essential for production of the staphyloxanthin pigment, but was unable to induce pigment production on its own. Interestingly, staphyloxanthin production was induced by a *P. aeruginosa* co-isolate (Antonic et al., 2013). Furthermore, the pigment production in a characteristically golden-yellow *S. aureus* strain, which was co-isolated with the *Pseudomonas* strain and the white *S. aureus* variant, remained unaffected. However, this contradicts other studies that report an inhibition of *S. aureus* pigment production by pyocyanin and pyoverdin produced by *P. aeruginosa* (Biswas et al., 2009). Another result of this study (Antonic et al., 2013) that is in contradiction with other studies is that there was an unchanged expression of *sigB* that encodes the alternative transcription factor sigma B and which is previously reported to be upregulated in the presence of *P. aeruginosa* (Mitchell et al., 2010). The discrepancies in results between different studies indicate the importance of co-evolution and adaptation of the different isolates to each other and their environment. Adaptation of *S. aureus* to *P. aeruginosa* might lead to an expression pattern that is similar to a stress-resistant phenotype.

S. AUREUS SURVIVES IN THE PRESENCE OF *P. AERUGINOSA* AS THE SMALL COLONY VARIANT PHENOTYPE

As a defense mechanism, *S. aureus* has also devised strategies to survive in the presence of *P. aeruginosa*. One of these is the switch to the SCV, a well-characterized phenotype detected in various diseases, including CF and device-related infections (Proctor et al., 2006). SCVs appear as small, smooth colonies on a culture plate and grow significantly slower compared to wild type colonies. The SCV phenotype might appear naturally and is caused by a defective or inhibited electron transport pathway that switches *S. aureus* to a fermentative growth state. In addition to a decreased growth rate, SCVs also demonstrate decreased ATP yield, decreased pigmentation and often hemin or menadione auxotrophy (Proctor et al., 2006; Biswas et al., 2009). Remarkably, the switch to a SCV phenotype increases survival of *S. aureus* in unfavorable conditions as it exhibits an increased aminoglycoside resistance, biofilm formation, and intracellular survival (Hoffman et al., 2006; Proctor et al., 2006; Biswas et al., 2009; Atalla et al., 2011). Prolonged co-culture with *P. aeruginosa* or exposure to pure HQNO leads to a high proportion of stable *S. aureus* SCVs, an effect that is increased by the presence of aminoglycosides (Hoffman et al., 2006). It has also been proposed that the reason why *S. aureus* and *P. aeruginosa* are not frequently detected together in diagnostic cultures of sputum of CF patients is because of the existence of *S. aureus* as SCVs that are more difficult to detect due to their small size and fastidious growth requirements (Proctor et al., 2006; Atalla et al., 2011).

SCV Induction by *P. aeruginosa* Is Sigma B Dependent

After the induction of the SCV phenotype during exposure to HQNO, the expression of three main regulatory mechanisms of virulence and biofilm formation is altered. First, the alternative transcription factor sigma B (SigB) is upregulated (Mitchell et al., 2010). SigB regulates the general stress response of Gram-positive bacteria, repressing the expression of most exoenzymes and toxins, stimulating the expression of adhesins and promoting the persistence of *S. aureus* in host cells (van Schaik and Abee, 2005; Atalla et al., 2011). Second, stimulation of SigB was shown to stimulate the expression of the Staphylococcal accessory regulator SarA, which modulates the expression of the pore-forming toxin α-hemolysin (*hla*) and increases biofilm formation (Valle et al., 2003; Oscarsson et al., 2006; Mitchell et al., 2010). In addition, SigB represses a third important regulator of *S. aureus* biofilm formation, the accessory gene regulator (*agr*) system, which induces biofilm dispersal thereby decreasing the total biomass and increases the expression of *hla* (Boles and Horswill, 2008; Mitchell et al., 2010; Atalla et al., 2011). In conclusion, HQNO reduces the production of the toxin *hla* by increasing the expression of SigB, leading to an increased expression of SarA and a decreased expression of *agr*. Reduced expression of toxins helps *S. aureus* to remain intracellular and thus increase its chances of survival in the human host. The stimulation of sarA by upregulation of SigB might be counteracted by long chain AHLs,

which inhibit sarA (**Figure 2**, right lower panel). The net effect is probably dependent on the *Pseudomonas* strain involved since the production of both factors might be variable between isolates (Qazi et al., 2006; Fugère et al., 2014).

P. AERUGINOSA AND *S. AUREUS* IN DUAL SPECIES BIOFILMS

During dual species biofilm formation, the balance of attacking, evading and counter-attacking is even more important and the properties of each strain as well as some environmental factors will determine if a dual species biofilm will be formed. For example, the presence of environmental selection pressure, like antibiotics or the host immune system, stimulates a more synergistic relationship and biofilm formation as the tolerance of *S. aureus* to antibiotics is significantly higher during co-culture with *P. aeruginosa* (DeLeon et al., 2014; Kumar and Ting, 2015). In the following paragraphs we describe how certain extracellular factors influence the structure, characteristics and composition of the dual species biofilms (**Figure 3**).

P. aeruginosa Extracellular Polysaccharides Are Important in the Formation of Multi-Species Biofilms

P. aeruginosa produces three main exopolysaccharides (EPS): alginate, Pel, and Psl, which form the extracellular matrix in the biofilm and have a structural and protective function (Leid et al., 2005; Ryder et al., 2007; Colvin et al., 2011, 2012). Pel and Psl are the main EPS in non-mucoid strains (Colvin et al., 2012). The Psl polysaccharide is recently identified as repeating units of glucose-, mannose-, and rhamnose-sugars and is mainly produced during the attachment phase of the biofilm (Colvin et al., 2012). Psl-positive strains have an elastic matrix with highly effective cross-linking of the matrix components (Chew et al., 2014). Pel is glucose-rich and is mainly involved in pellicle formation and later stages of biofilm formation (Colvin et al., 2012). Contrary to Psl, Pel dominant strains form loose biofilm structures since Pel reduces the effective cross-linking in the matrix network (**Figure 3**; Chew et al., 2014). Consequently, Pel-mediated loosening of the *P. aeruginosa* biofilm allows *S. aureus* to infiltrate into the biofilm and form multi-species biofilms (Chew et al., 2014). In contrast, the role of Psl in multi-species biofilm formation is not very clear. Chew et al showed that co-culture of a Psl-positive strain (PAO1) and *S. aureus* resulted in separated microcolonies without much association between both species (Chew et al., 2014). Billings et al., on the other hand, showed that *S. aureus* was incorporated in the air-liquid interface of a Psl producing *P. aeruginosa* biofilm. Both studies use the same biofilm assay and the same *P. aeruginosa* strains, PAO1 and mutants derived from PAO1, but different *S. aureus* strains. Because the biofilm structure and dual species interactions are dependent on both *P. aeruginosa* and the *Staphylococcus* strains, this might explain the discrepancy between the studies. Nonetheless, both studies concluded that the EPS provides protection against antibiotics to all inhabitants of the biofilm, even the non-producers, although the biofilm as a whole is weakened (Billings et al., 2013). More specifically, Psl functions as a protective barrier against the antibiotics colistin and polymyxin B, whereas Pel offers a protective barrier against aminoglycosides (**Figure 3**; Colvin et al., 2011; Billings et al., 2013). These findings indicate that a minimum amount of EPS per cell present in the biofilm is needed for optimal protection against antibiotics (Billings et al., 2013). These data suggests, if *S. aureus* is able to survive killing by *P. aeruginosa* and to co-exist in a multi-species biofilm, it benefits from the antimicrobial barrier formed by the *P. aeruginosa* matrix components. However, the third EPS, alginate, was not shown to have an effect on *S. aureus* and *S. epidermidis* biofilm formation. Alginate is mainly associated with chronic infections as its overproduction leads to the mucoid phenotype that frequently arises during long-term CF lung infection (Ryder et al., 2007). The switch to a mucoid phenotype contributes to the establishment of chronic colonization since alginate offers structural protection against uptake by macrophages and antimicrobials by forming a barrier limiting the penetration of antimicrobials, macrophages and macrophage-derived products, such as the pro-phagocytic cytokine IFN-γ (Leid et al., 2005; Ryder et al., 2007). Moreover, Leid et al. suggest that alginate might cause the transition from acute to chronic infection by limiting IFN-mediated clearance by macrophages, which is the main mechanism of bacterial clearance during acute infection (Leid et al., 2005).

S. aureus Protein a Binds to Psl and Type IV Pili of *P. aeruginosa*

In addition to EPS, other extracellular factors are important in dual species biofilm formation. Yang et al showed that only *P. aeruginosa* strains producing type IV pili co-aggregate with *S. aureus* in microcolonies (Yang et al., 2011). Type IV pili probably facilitate biofilm formation by binding to extracellular DNA (eDNA), which is derived from dead bacteria and part of the biofilm matrix (Yang et al., 2011). *P. aeruginosa* strains defective for the production of type IV pili or even treating the biofilm with DNAse I was shown to reduce the growth of mixed-species microcolonies (Yang et al., 2011). Furthermore, using a single *S. aureus* laboratory strain, Armbruster et al. showed inhibition of surface attachment of some *P. aeruginosa* clinical isolates due to the secretion of protein A (SpA) by *S. aureus*. SpA is a cell-wall associated extracellular adhesive protein of *S. aureus* that mediates biofilm formation and disrupts phagocytosis (by binding to the Fc portion of IgG antibodies) and its secretion was shown to be increased in artificial sputum (Armbruster et al., 2016). Secreted SpA was shown to specifically bind both Psl and type IV pili of *P. aeruginosa*, stressing the importance of these two molecules in multispecies interactions (**Figure 3**; Armbruster et al., 2016). In a Psl producing *P. aeruginosa*, all SpA seem to bind to the Psl, leaving the type IV pili free to mediate biofilm formation. In absence of Psl, SpA binds to the PilA component of type IV pili and inhibits adhesion of *P. aeruginosa* (Armbruster et al., 2016). Furthermore, SpA seems to protect *P. aeruginosa* from phagocytosis, as the Psl-SpA complex is no longer recognized by anti-Psl IgG antibodies. SpA

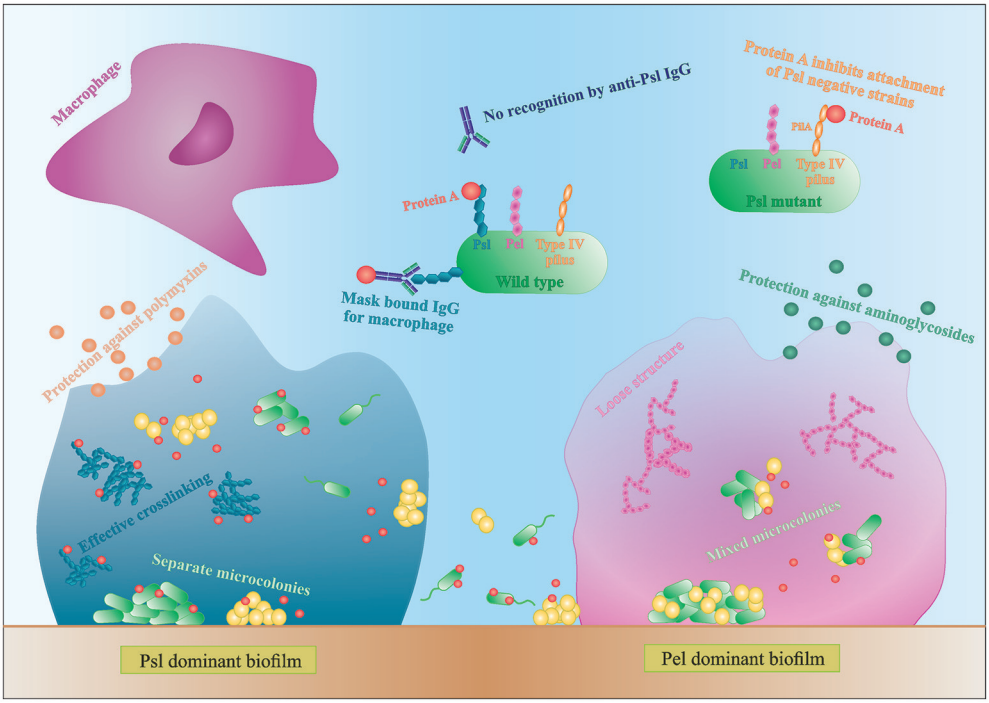


FIGURE 3 | Interactions in mixed species biofilms. Differences in biofilm formation by *S. aureus* with a Psl- or Pel-dominant *P. aeruginosa* strain. IgG, immunoglobulin G.

can also bind to the Fc domain of anti-Psl IgG antibodies and prevent recognition by neutrophils (**Figure 3**; Armbruster et al., 2016).

P. AERUGINOSA AND OTHER STAPHYLOCOCCI

P. aeruginosa Induces Biofilm Dispersal in *S. epidermidis*

The antistaphylococcal molecules produced by *P. aeruginosa* are also active against other staphylococci, including *S. epidermidis*, although some are more resistant to killing compared to *S. aureus*. *P. aeruginosa* was shown to effectively inhibit and disrupt established *S. epidermidis* biofilms and induce detachment without killing during dual species biofilm formation (Qin et al., 2009; Pihl et al., 2010a,b). After co-inoculation in equal proportions, *P. aeruginosa* and *S. epidermidis* could coexist for up to 18 h. After this time point, the *S. epidermidis* cells in the biofilm are lysed by *P. aeruginosa* (Pihl et al., 2010a). These data suggest that there are two stages in interactions between *P. aeruginosa* and *S. epidermidis*, the first includes the induction of detachment of viable *S. epidermidis* cells from the biofilm, while in the second stage cell lysis causes the total detachment (Pihl et al., 2010a). Similar to *S. aureus*, the effect of *P. aeruginosa* on *S. epidermidis* is strain dependent as some *P. aeruginosa* strains have a more pronounced effect on some *S. epidermidis* strains while others are more resistant to *P. aeruginosa* (Pihl et al., 2010b). Nevertheless, extracellular products that prevent initial attachment of some *S. epidermidis* strains to surfaces might be an interesting option for the development of coatings for indwelling medical devices, like peritoneal dialysis catheters (Pihl et al., 2013). Moreover, in this model, *P. aeruginosa* supernatant components replaced serum proteins on the catheter surface and reduced *S. epidermidis* attachment (Pihl et al., 2013). In addition, exposure of a *S. epidermidis* biofilm on a catheter to *P. aeruginosa* supernatant also caused dispersal of *S. epidermidis* (Pihl et al., 2013). The dispersed cells are, however, not killed making it less suitable as a treatment option and only interesting as a prevention strategy.

Yayurea A and B from the *S. intermedius* Group Are Quorum-Quenching Molecules Which Provide Protection against Gram Negative Bacteria

P. aeruginosa is originally an environmental bacterium and shares a niche with many other, non-pathogenic staphylococci like the *Staphylococcus intermedius* group consisting of *S. delphini, S. intermedius, S. lutrae, S. pseudointermedius* and *S. schleiferi*. All are common colonizers of various animals and rarely occur in humans (Simou et al., 2005; Ruscher et al., 2009). This group of staphylococci produces two low molecular weight compounds, yayurea A and B, that inhibit the production of quorum-sensing regulated products in Gram negative bacteria and provide protection against extracellular compounds

produced by *P. aeruginosa* (Chu et al., 2013). For example, the growth of *S. delphini* is not suppressed by respiratory toxins during co-culture with *P. aeruginosa.* Moreover, *S. delphini* is able to completely inhibit the production of pyocyanin (Chu et al., 2013). The quorum-quenching effect of yayurea A and B covers a broad spectrum of Gram negative bacteria, including *P. aeruginosa, Serratia marcescens, Vibrio harveyi,* and *Chromobacterium subtsugae* (Chu et al., 2013). Quenching of the quorum-sensing system of these Gram negative bacteria does not kill them but rather maintains their physiological state as if the cell density is low, even though density is in fact high. This increases the chances of survival of the staphylococci since toxin production usually begins at high cell density (Chu et al., 2013). Surprisingly, other staphylococci seem to be resistant to both molecules, even though they are not producers (Chu et al., 2013). Interestingly, *S. aureus* is protected from killing by *P. aeruginosa* when yayurea A and B are added to the medium without the former having to undergo physiological changes (SCV formation), amd represent promising candidates for inhibition studies of P. aeruginosa virulence and biofilm formation.

Variations in CydAB from *S. carnosus* Provides Protection Against Killing by *P. aeruginosa*

In addition to the *S. intermedius* group, several other non-pathogenic staphylococci (*S. carnosus*, *S. pisciferm*entans, and *S. simulans*) seem to be resistant to respiratory toxins secreted by *P. aeruginosa* due to alterations in the cydAB genes. These genes encode the two subunits of cytochrome bd quinol oxidase, of which homologs are also present in the genomes of *S. aureus* and *S. epidermidis* (Voggu et al., 2006). However, only the cytochrome bd quinol oxidase of the first group is resistant to the respiratory toxin, pyocyanin (Voggu et al., 2006). Furthermore, cloning of the *S. carnosus* cydAB cluster into *S. aureus* confers resistance to respiratory inhibitors produced by *P. aeruginosa* (Voggu et al., 2006). Further research showed that, whereas the CydA subunit is more conserved in staphylococci, CydB underwent a microevolution with relatively higher identity within than between the groups of pathogenic and non-pathogenic staphylococci (Voggu et al., 2006). This asymmetric evolution of CydB could be explained by the fact that the non-pathogenic staphylococci frequently inhabit the same environment as *Pseudomonas* spp. and were therefore selected for a higher resistance to respiratory toxins.

CONCLUDING REMARKS

Both *P. aeruginosa* and staphylococci are highly versatile organisms, which readily adapt to a wide variety of environments and stress factors. In the first glance, these bacteria seem to have an antagonistic relationship as *P. aeruginosa* produces a wide variety of molecules inhibiting staphylococci and frequently outcompetes *S. aureus* and *S. epidermidis* during co-culture. This antagonistic behavior is mainly shown during planktonic growth and under traditional culture conditions, where no host factors or antibiotics are present. However, under some *in vitro* and *in vivo* circumstances, both bacteria are able to co-exist and form dual species biofilms. These circumstances are dependent on a combination of strain-dependent properties of both species and the presence or absence of certain environmental factors like antibiotics or host factors, the sum of which might tip the balance toward either killing or co-existence. The presence of some sort of selection pressure or presence of a preformed matrix seems to favor dual species biofilm formation whereas planktonic co-culture without selection pressure leads to domination of *P. aeruginosa*. Interspecies competition often leads to an increased production of virulence factors in both *P. aeruginosa* and *S. aureus*, which are also harmful to the human host. In addition, escaping the antistaphylococcal compounds results in a more stress-resistant phenotype of *S. aureus*, which is more difficult to be cleared by the immune system, to be eradicated by antibiotics and to be detected in diagnostic cultures. Furthermore, the presence of an extracellular matrix was shown to be beneficial for all biofilm inhabitants, providing protection against classical antibiotics and the host immune system, although the exact composition might be variable depending on the species present and the environment. The role of these matrix components (exopolysaccharides, eDNA, matrix proteins, host-derived factors etc.) in interspecies interactions and their role in disease pathogenesis provides an exciting opportunity for future research toward better patient care. When strains are co-existing for a longer time, they might evolve to a phenotype that is better adapted to the presence of the other. For example, non-pathogenic staphylococci that are frequently encountering *P. aeruginosa* have developed strategies to continue growing in the presence of *P. aeruginosa* antistaphylcoccal compounds, indicating parallel evolution. Moreover, *S. aureus* and *P. aeruginosa* strains isolated from the same chronic CF lung infection are less sensitive to, and produce less, HQNO, respectively. This strain adaptation and the underestimation of the co-existence of *P. aeruginosa* and *S. aureus* might still have a large impact on the clinical outcome of a patient and therefore should be a subject of continuing investigation.

AUTHOR CONTRIBUTIONS

AH collected literature. All authors contributed in drafting the review.

FUNDING

AH was supported by funding from the Innovative Medicines Initiative project COMBACTE-MAGNET (Combatting Bacterial Resistance in Europe—Molecules Against Gram Negative Infections) (IMI Grant Agreement No 115737-2) and funding from Research Foundation Flanders (FWO-F, G.0513.12).

REFERENCES

Antonic, V., Stojadinovic, A., Zhang, B., Izadjoo, M. J., and Alavi, M. (2013). *Pseudomonas aeruginosa* induces pigment production and enhances virulence in a white phenotypic variant of *Staphylococcus aureus*. *Infect. Drug Resist.* 6, 175–186. doi: 10.2147/IDR.S49039

Armbruster, C. R., Wolter, D. J., Mishra, M., Hayden, H. S., Radey, M. C., Merrihew, G., et al. (2016). *Staphylococcus aureus* protein A mediates interspecies interactions at the cell surface of *Pseudomonas aeruginosa*. *MBio* 7:e00538-16. doi: 10.1128/mBio.00538-16

Atalla, H., Gyles, C., and Mallard, B. (2011). *Staphylococcus aureus* small colony variants (SCVs) and their role in disease. *Anim. Health Res. Rev.* 12, 33–45. doi: 10.1017/S1466252311000065

Baldan, R., Cigana, C., Testa, F., Bianconi, I., De Simone, M., Pellin, D., et al. (2014). Adaptation of *Pseudomonas aeruginosa* in Cystic Fibrosis airways influences virulence of *Staphylococcus aureus in vitro* and murine models of co-infection. *PLoS ONE* 9:89614. doi: 10.1371/journal.pone.0089614

Barequet, I. S., Habot-Wilner, Z., Mann, O., Safrin, M., Ohman, D. E., Kessler, E., et al. (2009). Evaluation of *Pseudomonas aeruginosa* staphylolysin (LasA protease) in the treatment of methicillin-resistant *Staphylococcus aureus* endophthalmitis in a rat model. *Graefes Arch. Clin. Exp. Ophthalmol.* 247, 913–917. doi: 10.1007/s00417-009-1061-2

Billings, N., Millan, M., Caldara, M., Rusconi, R., Tarasova, Y., Stocker, R., et al. (2013). The extracellular matrix Component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog.* 9:e1003526. doi: 10.1371/journal.ppat.1003526

Biswas, L., Biswas, R., Schlag, M., Bertram, R., and Gotz, F. (2009). Small-colony variant selection as a survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 75, 6910–6912. doi: 10.1128/aem.01211-09

Boles, B. R., and Horswill, A. R. (2008). Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog.* 4:e1000052. doi: 10.1371/journal.ppat.1000052

Burmolle, M., Ren, D., Bjarnsholt, T., and Sorensen, S. J. (2014). Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* 22, 84–91. doi: 10.1016/j.tim.2013.12.004

Cystic Fibrosis Foundation (2014). 2013 patient registry annual data report. Bathesda, MD: Cystic Fibrosis Foundation.

Cystic Fibrosis Foundation Patient Registry (2012). 2011 annual data report. Bathesda, MD: Cystic Fibrosis Foundation.

Caldwell, C. C., Chen, Y., Goetzmann, H. S., Hao, Y., Borchers, M. T., Hassett, D. J., et al. (2009). *Pseudomonas aeruginosa* exotoxin pyocyanin causes cystic fibrosis airway pathogenesis. *Am. J. Pathol.* 175, 2473–2488. doi: 10.2353/ajpath.2009.090166

Chew, S. C., Kundukad, B., Seviour, T., van der Maarel, J. R., Yang, L., Rice, S. A., et al. (2014). Dynamic remodeling of microbial biofilms by functionally distinct exopolysaccharides. *MBio* 5, e01536–e01514. doi: 10.1128/mBio.01536-14

Chu, Y. Y., Nega, M., Wolfle, M., Plener, L., Grond, S., Jung, K., et al. (2013). A new class of quorum quenching molecules from Staphylococcus species affects communication and growth of gram-negative bacteria. *PLoS Pathog.* 9:e1003654. doi: 10.1371/journal.ppat.1003654

Colvin, K. M., Gordon, V. D., Murakami, K., Borlee, B. R., Wozniak, D. J., Wong, G. C., et al. (2011). The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog.* 7:e1001264. doi: 10.1371/journal.ppat.1001264

Colvin, K. M., Irie, Y., Tart, C. S., Urbano, R., Whitney, J. C., Ryder, C., et al. (2012). The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environ. Microbiol.* 14, 1913–1928. doi: 10.1111/j.1462-2920.2011.02657.x

Dalton, T., Dowd, S. E., Wolcott, R. D., Sun, Y., Watters, C., Griswold, J. A., et al. (2011). An *in vivo* polymicrobial biofilm wound infection model to study interspecies interactions. *PLoS ONE* 6:e27317. doi: 10.1371/journal.pone.0027317

Davies, D. G., and Marques, C. N. (2009). A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. *J. Bacteriol.* 191, 1393–1403. doi: 10.1128/jb.01214-08

DeLeon, S., Clinton, A., Fowler, H., Everett, J., Horswill, A. R., and Rumbaugh, K. P. (2014). Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an *in vitro* wound model. *Infect. Immun.* 82, 4718–4728. doi: 10.1128/iai.02198-14

Deziel, E., Lepine, F., Milot, S., He, J., Mindrinos, M. N., Tompkins, R. G., et al. (2004). Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1339–1344. doi: 10.1073/pnas.0307694100

Diggle, S. P., Matthijs, S., Wright, V. J., Fletcher, M. P., Chhabra, S. R., Lamont, I. L., et al. (2007). The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem. Biol.* 14, 87–96. doi: 10.1016/j.chembiol.2006.11.014

Donlan, R. M., and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167–193. doi: 10.1128/CMR.15.2.167-193.2002

Duan, K., Dammel, C., Stein, J., Rabin, H., and Surette, M. G. (2003). Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol. Microbiol.* 50, 1477–1491. doi: 10.1046/j.1365-2958.2003.03803.x

Falcon, M. A., Mansito, T. B., Carnicero, A., and Gutierrez-Navarro, A. M. (1989). L-form-like colonies of *Staphylococcus aureus* induced by an extracellular lytic enzyme from *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 27, 1650–1654.

Fazli, M., Bjarnsholt, T., Kirketerp-MØller, K., JØrgensen, B., Andersen, A. S., Krogfelt, K. A., et al. (2009). Nonrandom distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds. *J. Clin. Microbiol.* 47, 4084–4089. doi: 10.1128/jcm.01395-09

Federle, M. J., and Bassler, B. L. (2003). Interspecies communication in bacteria. *J. Clin. Invest.* 112, 1291–1299. doi: 10.1172/jci20195

Flemming, H. C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi: 10.1038/nrmicro2415

Fugére, A., Lalonde Séguin, D., Mitchell, G., Déziel, E., Dekimpe, V., Cantin, A. M., et al. (2014). Interspecific small molecule interactions between clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from adult cystic fibrosis patients. *PLoS ONE* 9:e86705. doi: 10.1371/journal.pone.0086705

Gibson, J., Sood, A., and Hogan, D. A. (2009). *Pseudomonas aeruginosa*-Candida albicans interactions: localization and fungal toxicity of a phenazine derivative. *Appl. Environ. Microbiol.* 75, 504–513. doi: 10.1128/AEM.01037-08

Haba, E., Pinazo, A., Jauregui, O., Espuny, M. J., Infante, M. R., and Manresa, A. (2003). Physicochemical characterization and antimicrobial properties of rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044. *Biotechnol. Bioeng.* 81, 316–322. doi: 10.1002/bit.10474

Hall-Stoodley, L., and Stoodley, P. (2005). Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbiol.* 13, 7–10. doi: 10.1016/j.tim.2004.11.004

Hall-Stoodley, L., Costerton, J. W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108. doi: 10.1038/nrmicro821

Harrison, F. (2007). Microbial ecology of the cystic fibrosis lung. *Microbiology* 153, 917–923. doi: 10.1099/mic.0.2006/004077-0

Hauser, A. R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat. Rev. Microbiol.* 7, 654–665. doi: 10.1038/nrmicro2199

Hauser, A. R., Jain, M., Bar-Meir, M., and McColley, S. A. (2011). Clinical significance of microbial infection and adaptation in cystic fibrosis. *Clin. Microbiol. Rev.* 24, 29–70. doi: 10.1128/CMR.00036-10

Hoffman, L. R., Deziel, E., D'Argenio, D. A., Lepine, F., Emerson, J., McNamara, S., et al. (2006). Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19890–19895. doi: 10.1073/pnas.0606756104

Hotterbeekx, A., Xavier, B. B., Bielen, K., Lammens, C., Moons, P., Schepens, T., et al. (2016). The endotracheal tube microbiome associated with *Pseudomonas aeruginosa* or *Staphylococcus epidermidis*. *Sci. Rep.* 6:36507. doi: 10.1038/srep36507

Hubert, D., Réglier-Poupet, H., Sermet-Gaudelus, I., Ferroni, A., Le Bourgeois, M., Burgel, P. R., et al. (2013). Association between *Staphylococcus aureus* alone or combined with *Pseudomonas aeruginosa* and the clinical condition of patients with cystic fibrosis. *J. Cyst. Fibros.* 12, 497–503. doi: 10.1016/j.jcf.2012.12.003

Jefferson, K. K. (2004). What drives bacteria to produce a biofilm? *FEMS Microbiol. Lett.* 236, 163–173. doi: 10.1111/j.1574-6968.2004.tb09643.x

Jensen, P. Ø., Bjarnsholt, T., Phipps, R., Rasmussen, T. B., Calum, H., Christoffersen, L., et al. (2007). Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology* 153, 1329–1338. doi: 10.1099/mic.0.2006/003863-0

Jimenez, P. N., Koch, G., Thompson, J. A., Xavier, K. B., Cool, R. H., and Quax, W. J. (2012). The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.* 76, 46–65. doi: 10.1128/MMBR.05007-11

Kessler, E., Safrin, M., Olson, J. C., and Ohman, D. E. (1993). Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. *J. Biol. Chem.* 268, 7503–7508.

Kirketerp-MØller, K., Jensen, P. Ø., Fazli, M., Madsen, K. G., Pedersen, J., Moser, C., et al. (2008). Distribution, organization, and ecology of bacteria in chronic wounds. *J. Clin. Microbiol.* 46, 2717–2722. doi: 10.1128/jcm.00501-08

Korgaonkar, A. K., and Whiteley, M. (2011). *Pseudomonas aeruginosa* enhances production of an antimicrobial in response to N-acetylglucosamine and peptidoglycan. *J. Bacteriol.* 193, 909–917. doi: 10.1128/JB.01175-10

Korgaonkar, A., Trivedi, U., Rumbaugh, K. P., and Whiteley, M. (2013). Community surveillance enhances *Pseudomonas aeruginosa* virulence during polymicrobial infection. *Proc. Natl. Acad. Sci. U.S.A.* 110, 1059–1064. doi: 10.1073/pnas.1214550110

Kumar, A., and Ting, Y. P. (2015). Presence of *Pseudomonas aeruginosa* influences biofilm formation and surface protein expression of *Staphylococcus aureus*. *Environ. Microbiol.* 17, 4459–4468. doi: 10.1111/1462-2920.12890

Leid, J. G., Willson, C. J., Shirtliff, M. E., Hassett, D. J., Parsek, M. R., and Jeffers, A. K. (2005). The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-γ-mediated macrophage killing. *J. Immunol.* 175, 7512–7518. doi: 10.4049/jimmunol.175.11.7512

Li, H., Li, X., Wang, Z., Fu, Y., Ai, Q., Dong, Y., et al. (2015). Autoinducer-2 regulates *Pseudomonas aeruginosa* PAO1 biofilm formation and virulence production in a dose-dependent manner. *BMC Microbiol.* 15:192. doi: 10.1186/s12866-015-0529-y

Li, Y. H., and Tian, X. (2012). Quorum sensing and bacterial social interactions in biofilms. *Sensors* 12, 2519–2538. doi: 10.3390/s120302519

Lightbown, J. W., and Jackson, F. L. (1956). Inhibition of cytochrome systems of heart muscle and certain bacteria by the antagonists of dihydrostreptomycin: 2-alkyl-4-hydroxyquinoline N-oxides. *Biochem. J.* 63, 130–137. doi: 10.1042/bj0630130

Machan, Z. A., Pitt, T. L., White, W., Watson, D., Taylor, G. W., Cole, P. J., et al. (1991). Interaction between *Pseudomonas aeruginosa* and *Staphylococcus aureus*: description of an anti-staphylococcal substance. *J. Med. Microbiol.* 34, 213–217. doi: 10.1099/00222615-34-4-213

Machan, Z. A., Taylor, G. W., Pitt, T. L., Cole, P. J., and Wilson, R. (1992). 2-Heptyl-4-hydroxyquinoline N-oxide, an antistaphylococcal agent produced by *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 30, 615–623. doi: 10.1093/jac/30.5.615

Mashburn, L. M., Jett, A. M., Akins, D. R., and Whiteley, M. (2005). *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during *in vivo* coculture. *J. Bacteriol.* 187, 554–566. doi: 10.1128/JB.187.2.554-566.2005

Mitchell, G., Séguin, D. L., Asselin, A. E., Deziel, E., Cantin, A. M., Frost, E. H., et al. (2010). *Staphylococcus aureus* sigma B-dependent emergence of small-colony variants and biofilm production following exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-N-oxide. *BMC Microbiol.* 10:33. doi: 10.1186/1471-2180-10-33

Oscarsson, J., Kanth, A., Tegmark-Wisell, K., and Arvidson, S. (2006). SarA is a repressor of hla (α-hemolysin) transcription in *Staphylococcus aureus*: its apparent role as an activator of hla in the prototype strain NCTC 8325 depends on reduced expression of sarS. *J. Bacteriol.* 188, 8526–8533. doi: 10.1128/JB.00866-06

Palmer, K. L., Mashburn, L. M., Singh, P. K., and Whiteley, M. (2005). Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J. Bacteriol.* 187, 5267–5277. doi: 10.1128/jb.187.15.5267-5277.2005

Pastar, I., Nusbaum, A. G., Gil, J., Patel, S. B., Chen, J., Valdes, J., et al. (2013). Interactions of methicillin resistant *Staphylococcus aureus* USA300 and *Pseudomonas aeruginosa* in polymicrobial wound infection. *PLoS ONE* 8:e56846. doi: 10.1371/journal.pone.0056846

Petrova, O. E., and Sauer, K. (2016). Escaping the biofilm in more than one way: desorption, detachment or dispersion. *Curr. Opin. Microbiol.* 30, 67–78. doi: 10.1016/j.mib.2016.01.004

Pihl, M., Arvidsson, A., Skepö, M., Nilsson, M., Givskov, M., Tolker-Nielsen, T., et al. (2013). Biofilm formation by *Staphylococcus epidermidis* on peritoneal dialysis catheters and the effects of extracellular products from *Pseudomonas aeruginosa*. *Pathog. Dis.* 67, 192–198. doi: 10.1111/2049-632X.12035

Pihl, M., Chavez de Paz, L. E., Schmidtchen, A., Svensater, G., and Davies, J. R. (2010b). Effects of clinical isolates of *Pseudomonas aeruginosa* on *Staphylococcus epidermidis* biofilm formation. *FEMS Immunol. Med. Microbiol.* 59, 504–512. doi: 10.1111/j.1574-695X.2010.00707.x

Pihl, M., Davies, J. R., Chavez de Paz, L. E., and Svensater, G. (2010a). Differential effects of *Pseudomonas aeruginosa* on biofilm formation by different strains of *Staphylococcus epidermidis*. *FEMS Immunol. Med. Microbiol.* 59, 439–446. doi: 10.1111/j.1574-695X.2010.00697.x

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M., et al. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* 4, 295–305. doi: 10.1038/nrmicro1384

Qazi, S., Middleton, B., Muharram, S. H., Cockayne, A., Hill, P., O'Shea, P., et al. (2006). N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in *Staphylococcus aureus*. *Infect. Immun.* 74, 910–919. doi: 10.1128/IAI.74.2.910-919.2006

Qin, Z., Yang, L., Qu, D., Molin, S., and Tolker-Nielsen, T. (2009). *Pseudomonas aeruginosa* extracellular products inhibit staphylococcal growth, and disrupt established biofilms produced by *Staphylococcus epidermidis*. *Microbiology* 155, 2148–2156. doi: 10.1099/mic.0.028001-0

Rezzonico, F., Smits, T. H., and Duffy, B. (2012). Detection of AI-2 receptors in genomes of Enterobacteriaceae suggests a role of type-2 quorum sensing in closed ecosystems. *Sensors* 12, 6645–6665. doi: 10.3390/s120506645

Rodrigues, L. R., Banat, I. M., van der Mei, H. C., Teixeira, J. A., and Oliveira, R. (2006). Interference in adhesion of bacteria and yeasts isolated from explanted voice prostheses to silicone rubber by rhamnolipid biosurfactants. *J. Appl. Microbiol.* 100, 470–480. doi: 10.1111/j.1365-2672.2005.02826.x

Ruscher, C., Lubke-Becker, A., Wleklinski, C. G., Soba, A., Wieler, L. H., and Walther, B. (2009). Prevalence of Methicillin-resistant *Staphylococcus pseudintermedius* isolated from clinical samples of companion animals and equidaes. *Vet. Microbiol.* 136, 197–201. doi: 10.1016/j.vetmic.2008.10.023

Ryder, C., Byrd, M., and Wozniak, D. J. (2007). Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr. Opin. Microbiol.* 10, 644–648. doi: 10.1016/j.mib.2007.09.010

Sagel, S. D., Gibson, R. L., Emerson, J., McNamara, S., Burns, J. L., Wagener, J. S., et al. (2009). Impact of Pseudomonas and Staphylococcus infection on inflammation and clinical status in young children with cystic fibrosis. *J. Pediatr.* 154, 183–188. doi: 10.1016/j.jpeds.2008.08.001

Seth, A. K., Geringer, M. R., Galiano, R. D., Leung, K. P., Mustoe, T. A., and Hong, S. J. (2012). Quantitative comparison and analysis of species-specific wound biofilm virulence using an *in vivo*, rabbit-ear model. *J. Am. Coll. Surg.* 215, 388–399. doi: 10.1016/j.jamcollsurg.2012.05.028

Sibley, C. D., Duan, K., Fischer, C., Parkins, M. D., Storey, D. G., Rabin, H. R., et al. (2008). Discerning the complexity of community interactions using a Drosophila model of polymicrobial infections. *PLoS Pathog.* 4:e1000184. doi: 10.1371/journal.ppat.1000184

Simou, C., Hill, P. B., Forsythe, P. J., and Thoday, K. L. (2005). Species specificity in the adherence of staphylococci to canine and human corneocytes: a preliminary study. *Vet. Dermatol.* 16, 156–161. doi: 10.1111/j.1365-3164.2005.00452.x

Soberón-Chávez, G., Lépine, F., and Déziel, E. (2005). Production of rhamnolipids by *Pseudomonas aeruginosa*. *Appl. Microbiol. Biotechnol.* 68, 718–725. doi: 10.1007/s00253-005-0150-3

Sotirova, A. V., Spasova, D. I., Galabova, D. N., Karpenko, E., and Shulga, A. (2008). Rhamnolipid-biosurfactant permeabilizing effects on gram-positive and gram-negative bacterial strains. *Curr. Microbiol.* 56, 639–644. doi: 10.1007/s00284-008-9139-3

Strateva, T., and Mitov, I. (2011). Contribution of an arsenal of virulence factors to pathogenesis of *Pseudomonas aeruginosa* infections. *Ann. Microbiol.* 61, 717–732. doi: 10.1007/s13213-011-0273-y

Tashiro, Y., Yawata, Y., Toyofuku, M., Uchiyama, H., and Nomura, N. (2013). Interspecies interaction between *Pseudomonas aeruginosa* and other microorganisms. *Microbes Environ.* 28, 13–24. doi: 10.1264/jsme2.ME12167

Toyofuku, M., Nakajima-Kambe, T., Uchiyama, H., and Nomura, N. (2010). The effect of a cell-to-cell communication molecule, Pseudomonas quinolone signal (PQS), produced by *P. aeruginosa* on other bacterial species. *Microbes Environ.* 25, 1–7. doi: 10.1264/jsme2.ME09156

Valle, J., Toledo-Arana, A., Berasain, C., Ghigo, J. M., Amorena, B., Penades, J. R., et al. (2003). SarA and not sigmaB is essential for biofilm development by *Staphylococcus aureus*. *Mol. Microbiol.* 48, 1075–1087. doi: 10.1046/j.1365-2958.2003.03493.x

van Schaik, W., and Abee, T. (2005). The role of sigmaB in the stress response of Gram-positive bacteria – targets for food preservation and safety. *Curr. Opin. Biotechnol.* 16, 218–224. doi: 10.1016/j.copbio.2005.01.008

Voggu, L., Schlag, S., Biswas, R., Rosenstein, R., Rausch, C., and Gotz, F. (2006). Microevolution of cytochrome bd oxidase in Staphylococci and its implication in resistance to respiratory toxins released by Pseudomonas. *J. Bacteriol.* 188, 8079–8086. doi: 10.1128/jb.00858-06

Wang, Z., Xiang, Q., Yang, T., Li, L., Yang, J., Li, H., et al. (2016). Autoinducer-2 of *Streptococcus mitis* as a target molecule to inhibit pathogenic multi-species biofilm formation *in vitro* and in an endotracheal intubation rat model. *Front. Microbiol.* 7:88. doi: 10.3389/fmicb.2016.00088

Watters, C., Fleming, D., Bishop, D., and Rumbaugh, K. P. (2016). Host responses to biofilm. *Prog. Mol. Biol. Transl. Sci.* 142, 193–239. doi: 10.1016/bs.pmbts.2016.05.007

Whiley, R. A., Sheikh, N. P., Mushtaq, N., Hagi-Pavli, E., Personne, Y., Javaid, D., et al. (2014). Differential potentiation of the virulence of the *Pseudomonas aeruginosa* cystic fibrosis liverpool epidemic strain by oral commensal Streptococci. *J. Infect. Dis.* 209, 769–780. doi: 10.1093/infdis/jit568

Williams, P., and Camara, M. (2009). Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr. Opin. Microbiol.* 12, 182–191. doi: 10.1016/j.mib.2009.01.005

Yang, L., Liu, Y., Markussen, T., HØiby, N., Tolker-Nielsen, T., and Molin, S. (2011). Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *FEMS Immunol. Med. Microbiol.* 62, 339–347. doi: 10.1111/j.1574-695X.2011.00849.x

Zezzi do Valle Gomes, M., and Nitschke, M. (2012). Evaluation of rhamnolipid and surfactin to reduce the adhesion and remove biofilms of individual and mixed cultures of food pathogenic bacteria. *Food Control* 25, 441–447. doi: 10.1016/j.foodcont.2011.11.025

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

REVIEW
published: 13 March 2017
doi: 10.3389/fcimb.2017.00055

Biology of *Acinetobacter baumannii*: Pathogenesis, Antibiotic Resistance Mechanisms, and Prospective Treatment Options

Chang-Ro Lee[1†], Jung Hun Lee[1†], Moonhee Park[1,2†], Kwang Seung Park[1], Il Kwon Bae[3], Young Bae Kim[4], Chang-Jun Cha[5], Byeong Chul Jeong[1] and Sang Hee Lee[1]*

[1] National Leading Research Laboratory of Drug Resistance Proteomics, Department of Biological Sciences, Myongji University, Yongin, South Korea, [2] DNA Analysis Division, Seoul Institute, National Forensic Service, Seoul, South Korea, [3] Department of Dental Hygiene, College of Health and Welfare, Silla University, Busan, South Korea, [4] Biotechnology Program, North Shore Community College, Danvers, MA, USA, [5] Department of Systems Biotechnology, College of Biotechnology and Natural Resources, Chung-Ang University, Anseong, South Korea

Acinetobacter baumannii is undoubtedly one of the most successful pathogens responsible for hospital-acquired nosocomial infections in the modern healthcare system. Due to the prevalence of infections and outbreaks caused by multi-drug resistant *A. baumannii*, few antibiotics are effective for treating infections caused by this pathogen. To overcome this problem, knowledge of the pathogenesis and antibiotic resistance mechanisms of *A. baumannii* is important. In this review, we summarize current studies on the virulence factors that contribute to *A. baumannii* pathogenesis, including porins, capsular polysaccharides, lipopolysaccharides, phospholipases, outer membrane vesicles, metal acquisition systems, and protein secretion systems. Mechanisms of antibiotic resistance of this organism, including acquirement of β-lactamases, up-regulation of multidrug efflux pumps, modification of aminoglycosides, permeability defects, and alteration of target sites, are also discussed. Lastly, novel prospective treatment options for infections caused by multi-drug resistant *A. baumannii* are summarized.

Keywords: antimicrobial resistance, *Acinetobacter baumannii*, treatment option, resistance mechanism, virulence factor

OPEN ACCESS

Edited by:
Ghassan M. Matar, American University of Beirut, Lebanon

Reviewed by:
Sang Sun Yoon, Yonsei University, South Korea
Ravi Jhaveri, University of North Carolina Hospitals, USA

***Correspondence:**
Sang Hee Lee
sangheelee@mju.ac.kr

† These authors have contributed equally to this work.

***Received:** 19 December 2016*
***Accepted:** 13 February 2017*
***Published:** 13 March 2017*

Citation:
Lee C-R, Lee JH, Park M, Park KS, Bae IK, Kim YB, Cha C-J, Jeong BC and Lee SH (2017) Biology of Acinetobacter baumannii: Pathogenesis, Antibiotic Resistance Mechanisms, and Prospective Treatment Options. Front. Cell. Infect. Microbiol. 7:55. doi: 10.3389/fcimb.2017.00055

INTRODUCTION

Acinetobacter spp. are glucose-non-fermentative, non-motile, non-fastidious, catalase-positive, oxidative-negative, aerobic Gram-negative coccobacilli (Lin and Lan, 2014). Due to clusters of closely related species, it is difficult to distinguish *Acinetobacter* taxonomy using phenotypic traits and chemotaxonomic methods. Because antibiotic susceptibility and clinical relevance are significantly different between different genomic species, exact identification of *Acinetobacter* species are required (Bergogne-Berezin and Towner, 1996; Dijkshoorn et al., 1996; Houang et al., 2003; Lee et al., 2007). Many genomic fingerprinting methods have been developed, including repetitive extragenic palindromic sequence-based polymerase chain reaction (rep-PCR), pulsed-field gel electrophoresis (PFGE), matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, ribotyping, amplified ribosomal DNA restriction analysis,

random amplified polymorphic DNA analysis, multilocus sequence typing (MLST), RNA spacer fingerprinting, amplified fragment length polymorphism analysis, and sequence analysis of 16S-23S rRNA intergene spacer regions or the *rpoB* and *gyrB* genes (Koeleman et al., 1998; Chang et al., 2005; La Scola et al., 2006; Croxatto et al., 2012; Higgins et al., 2012; Lee C. R. et al., 2015; Li X. M. et al., 2016).

Among *Acinetobacter* species, *Acinetobacter baumannii* is the most important member associated with hospital-acquired infections worldwide (Lin and Lan, 2014). This aerobic Gram-negative coccobacillus had been regarded as a low-grade pathogen, but it is a successful pathogen responsible for opportunistic infections of the skin, bloodstream, urinary tract, and other soft tissues (Peleg et al., 2008). Because many *A. baumannii* infections have suddenly been reported among veterans and soldiers who served in Iraq and Afghanistan (Centers for Disease and Prevention, 2004), *A. baumannii* is referred to as "Iraqibacter." Multidrug-resistant (MDR) *A. baumannii* has spread to civilian hospitals in part by cross-infection of injured military patients repatriated from war zones (Peleg et al., 2008). Most *A. baumannii* infections occur in critically ill patients in the intensive care unit (ICU) setting (Fournier and Richet, 2006) and account for up to 20% of infections in ICUs worldwide (Vincent et al., 2009). Furthermore, the frequency of community-acquired *A. baumannii* infections has been increasing gradually (Lin and Lan, 2014). Several virulence factors have been identified by genomic and phenotypic analyses, including outer membrane porins, phospholipases, proteases, lipopolysaccharides (LPS), capsular polysaccharides, protein secretion systems, and iron-chelating systems (Antunes et al., 2011; McConnell et al., 2013; Lin and Lan, 2014).

Many reports have shown that *A. baumannii* rapidly develops resistance to antimicrobials, and multidrug-resistant strains have been isolated (McConnell et al., 2013). The WHO declared that *A. baumannii* is one of the most serious ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) that effectively escape the effects of antibacterial drugs (Boucher et al., 2009). A number of *A. baumannii* resistance mechanisms are known, including enzymatic degradation of drugs, target modifications, multidrug efflux pumps, and permeability defects (Gordon and Wareham, 2010; Kim et al., 2012; Lin and Lan, 2014). In this review, we summarize the virulence factors of *A. baumannii*, antibiotic resistance mechanisms, and the therapeutic options available for treating *A. baumannii* infections. **Figure 1** depicts all the features described in this review.

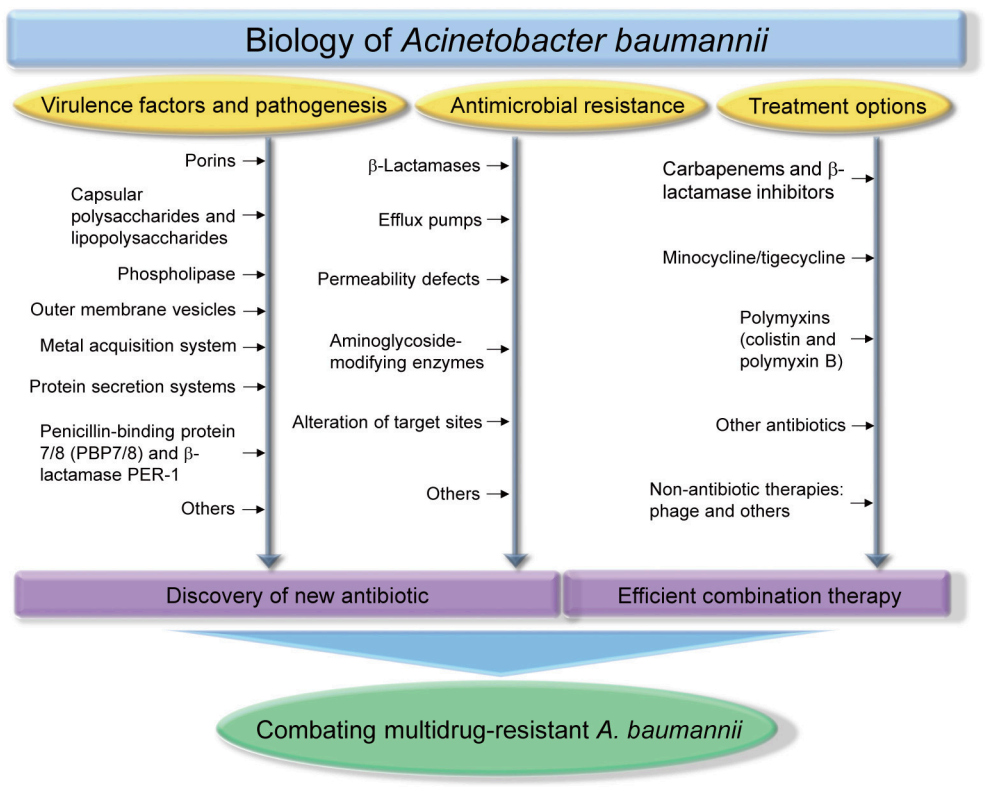


FIGURE 1 | Biology of *Acinetobacter baumannii*. Studies of virulence factors, pathogenesis, antimicrobial resistance, treatment options of *A. baumannii* will provide an important aid for discovering new antibiotics and determining efficient combination therapy, which are essential strategies for combating multidrug-resistant *A. baumannii* infections.

ACINETOBACTER BAUMANNII VIRULENCE FACTORS AND PATHOGENESIS

Although recent genomic and phenotypic analyses of *A. baumannii* have identified several virulence factors responsible for its pathogenicity, relatively few virulence factors have been identified in *A. baumannii*, compared to those in other Gram-negative pathogens (McConnell et al., 2013). The proposed *A. baumannii* virulence factors are summarized in **Table 1**.

Porins

Porins are outer membrane proteins associated with modulating cellular permeability. OmpA is a β-barrel porin and one of the most abundant porins in the outer membrane. In *A. baumannii*, OmpA is the very well-characterized virulence factor with a variety of interesting biological properties identified in *in vitro* model systems (Smith et al., 2007; McConnell et al., 2013). A random mutagenesis screen showed that the *A. baumannii ompA* mutant is defective in inducing apoptosis in human epithelial cells (Choi et al., 2005). Purified OmpA binds host epithelial cells, targets mitochondria, and induces apoptosis by releasing proapoptotic molecules, such as cytochrome c and apoptosis-inducing factor (Choi et al., 2005; Lee et al., 2010). Another study showed that OmpA translocates to the nucleus by a novel monopartite nuclear localization signal and induces cell death (Choi et al., 2008a). OmpA also plays a major role in adherence and invasion of epithelial cells by interacting with fibronectin (Choi et al., 2008b; Gaddy et al., 2009; Smani et al., 2012), and binds to factor H in human serum (Kim et al., 2009), which may allow *A. baumannii* to avoid complement-mediated killing. The

TABLE 1 | Identified virulence factors of *Acinetobacter baumannii*.

Virulence factor	Proposed role in pathogenesis	References
Porin (OmpA, Omp33-36, Omp22, CarO, OprD-like)	Adherence and invasion, induction of apoptosis, serum resistance, biofilm formation, persistence	Choi et al., 2005, 2008b; Gaddy et al., 2009; Kim et al., 2009; Lee et al., 2010; Fernandez-Cuenca et al., 2011; Smani et al., 2012, 2013; Rumbo et al., 2014; Wang et al., 2014; Huang et al., 2016
Capsular polysaccharide	Growth in serum, survival in tissue infection, biofilm formation	Russo et al., 2010; Iwashkiw et al., 2012; Lees-Miller et al., 2013
Lipopolysaccharide (LPS)	Serum resistance, survival in tissue infection, evasion of the host immune response	Luke et al., 2010; Lin et al., 2012; McQueary et al., 2012; McConnell et al., 2013
Phospholipase (PLC and PLD)	Serum resistance, invasion, *in vivo* survival	Camarena et al., 2010; Jacobs et al., 2010; Stahl et al., 2015; Fiester et al., 2016
Outer membrane vesicle (OMV)	Delivery of virulence factors, horizontal transfer of antibiotic resistance gene	Kwon et al., 2009; Jin et al., 2011; Rumbo et al., 2011; Moon et al., 2012; Jun et al., 2013; Li Z. T. et al., 2015
Iron acquisition system (acinetobactin and NfuA)	*In vivo* survival, persistence, killing of host cells	Gaddy et al., 2012; Penwell et al., 2012; Zimbler et al., 2012; Fiester et al., 2016; Megeed et al., 2016
Zinc acquisition system (ZnuABC and ZigA)	*In vivo* survival	Hood et al., 2012; Nairn et al., 2016
Manganese acquisition system (MumC and MumT)	*In vivo* survival	Juttukonda et al., 2016
Type II protein secretion system	*In vivo* survival	Johnson et al., 2015; Elhosseiny et al., 2016; Harding et al., 2016
Type VI protein secretion system	Killing of competing bacteria, host colonization	Carruthers et al., 2013; Wright et al., 2014; Jones et al., 2015; Repizo et al., 2015; Ruiz et al., 2015
Type V protein secretion system	Biofilm formation, adherence	Bentancor et al., 2012b
Penicillin-binding protein 7/8 and β-lactamase PER-1	Serum resistance, *in vivo* survival, adherence	Sechi et al., 2004; Russo et al., 2009
CipA	Serum resistance, invasion	Koenigs et al., 2016
Tuf	Serum resistance	Koenigs et al., 2015
RecA	*In vivo* survival	Aranda et al., 2011
SurA1	Serum resistance, *in vivo* survival	Liu D. et al., 2016
GigABCD	*In vivo* survival, killing of host cells	Gebhardt et al., 2015
UspA	*In vivo* survival, killing of host cells	Elhosseiny et al., 2015; Gebhardt et al., 2015
GacS and PaaE	Neutrophil influx	Cerqueira et al., 2014; Gebhardt et al., 2015; Bhuiyan et al., 2016
Pili	Adherence, biofilm formation	Tomaras et al., 2003, 2008
OmpR/EnvZ	Killing of host cells	Tipton and Rather, 2016
FhaBC	Adherence, killing of host cells	Perez et al., 2016
AbeD	Killing of host cells	Srinivasan et al., 2015

ompA gene is necessary for persistence of *A. baumannii* in the mouse lung (Wang et al., 2014).

Furthermore, OmpA is also involved in antimicrobial resistance of *A. baumannii* (Sugawara and Nikaido, 2012; Smani et al., 2014). The major *A. baumannii* porin is OmpA, which has 70-fold lower pore-forming activity than that of OmpF (Sugawara and Nikaido, 2012). Furthermore, disrupting the *ompA* gene significantly decreases the minimal inhibitory concentrations (MICs) of several antibiotics (chloramphenicol, aztreonam, and nalidixic acid), suggesting that OmpA participates in the extrusion of antibiotics from the periplasmic space through the outer membrane and couples with inner membrane efflux systems (Smani et al., 2014). OmpA enhances survival and persistence of *A. baumannii* by facilitating surface motility and biofilm formation (Gaddy et al., 2009; Clemmer et al., 2011; McConnell et al., 2013). OmpA also regulates biogenesis of outer membrane vesicles (Moon et al., 2012). These results suggest that the OmpA protein is an attractive target for developing novel antibiotics and prevention strategies. Two recent reports based on immuno-proteomics and reverse vaccinology suggested that OmpA is a potential vaccine candidate against *A. baumannii* (Fajardo Bonin et al., 2014; Hassan et al., 2016). Actually, the OmpA protein is immunogenic in healthy individuals and patients with *A. baumannii* invasive infections (Zhang et al., 2016). In a mouse model of *A. baumannii* infection, mice immunized with OmpA had a significantly higher survival rate than that of control mice (Luo et al., 2012; Lin L. et al., 2013; Zhang et al., 2016).

The 33- to 36-kDa Omp protein (Omp33-36), which acts as a water passage channel, is another outer membrane porin associated with *A. baumannii* cytotoxicity (Smani et al., 2013; Rumbo et al., 2014). The *omp33-36* deletion strain significantly reduces adherence and invasion of human lung epithelial cells and cytotoxicity to these cells (Smani et al., 2013). Deletion of the *omp33-36* gene in a murine sepsis model attenuates lethality and reduces bacterial concentrations in the spleen and lungs (Smani et al., 2013). One study showed that purified Omp33-36 induces apoptosis in several different cell types, including immune and connective tissue cells, by activating caspases and modulating autophagy (Rumbo et al., 2014). Omp33-36 is also involved in antibiotic resistance. *A. baumannii* strain JC10/01 resistant to carbapenem antibiotics (imipenem and meropenem) exhibits loss of Omp33-36 and episomal expression of Omp33-36 in this strain clearly reduces the MICs of imipenem and meropenem (del Mar Tomas et al., 2005).

Omp22 has also been identified as a novel, conserved, and safe antigen for developing effective vaccines to control *A. baumannii* infections (Huang et al., 2016), although the contribution of Omp22 to *A. baumannii* pathogenicity has not been determined. Both active and passive immunizations with Omp22 increase the survival rates of mice, suppress bacterial burdens in the organs and peripheral blood, and reduce serum levels of inflammatory cytokines and chemokines (Huang et al., 2016). Other porins, such as carbapenem-associated outer membrane protein (CarO) and OprD-like, are also virulence-related factors associated with attenuated virulence in a mouse model (Fernandez-Cuenca et al., 2011).

Capsular Polysaccharides and Lipopolysaccharides (LPS)

Beyond OmpA, the *A. baumannii* envelope is associated with many factors that contribute to pathogenicity. Among these, capsular exopolysaccharides and LPS are *A. baumannii* pathogenicity factors. Notably, many isolates from patients with *A. baumannii* infections express surface capsular polysaccharides and contain a conserved gene cluster, called the K locus, which may determine production of capsular polysaccharides (Koeleman et al., 2001; Hu et al., 2013; Kenyon and Hall, 2013; Geisinger and Isberg, 2015). A random transposon screening to identify genes essential for growth in an inflammatory exudative fluid lead to the identification of the *ptk* and *epsA* genes, which are predicted to be required for capsule polymerization and assembly (Russo et al., 2010). The *ptk* and *epsA* mutants are deficient in capsule production and have a growth defect in human serum, resulting in a highly significant decrease in survival in soft tissue infection sites (Russo et al., 2010). Mutation in the *pglC* or *pglL* gene, which is responsible for synthesis of the *O*-pentasaccharide found on glycoproteins and capsular polysaccharides, also attenuate lethality in a mouse septicemia model and form abnormal biofilm structures (Iwashkiw et al., 2012; Lees-Miller et al., 2013). Therefore, capsular polysaccharides have been proposed to be a target for protective antibody-based interventions (passive immunization; Russo et al., 2013).

One study showed that capsular polysaccharides are involved in antimicrobial resistance of *A. baumannii* (Geisinger and Isberg, 2015). Mutants deficient in capsular polysaccharides have lower intrinsic resistance to peptide antibiotics. In addition, the presence of antibiotics induces hyperproduction of capsular polysaccharides (Geisinger and Isberg, 2015). Antibiotic-induced production of capsular polysaccharides increases resistance to killing by host complement and increases virulence in a mouse model of systemic infection (Geisinger and Isberg, 2015). That study also demonstrated that increased capsule production after exposure to an antibiotic depends on transcriptional increases in K locus gene expression, and that expression of K locus genes is regulated by the *bfmRS* two-component regulatory system (Geisinger and Isberg, 2015). *bfmR* is a gene essential for growth in human ascites, which is an *ex vivo* medium that reflects the infection environment (Umland et al., 2012), and is important for persistence in the lung in a murine pneumonia model (Wang et al., 2014). BfmS is also a virulence factor that plays an important role in biofilm formation, adherence to eukaryotic cells, and resistance to human serum (Liou et al., 2014). On report showed BfmR-mediated resistance to complement-mediated bactericidal activity and resistance to the clinically important antimicrobials (meropenem and colistin; Russo et al., 2016). However, that study suggested that BfmR effects are independent of capsular polysaccharide production. Therefore, the relationship between BfmRS and capsular polysaccharides must be described in more detail.

LPS is the major component of the outer leaflet of the outer membrane in most Gram-negative bacteria and is an immunoreactive molecule that induces release of tumor necrosis factor and interleukin 8 from macrophages in a Toll-like receptor 4 (TLR4)-dependent manner (Erridge et al., 2007). LPS is composed of an endotoxic lipid A moiety, an oligosaccharide core, and a repetitive O-antigen (Lee et al., 2013b). In *A. baumannii*, LPS plays a major role in virulence and survival of *A. baumannii* (Luke et al., 2010; Lin et al., 2012; McQueary et al., 2012). Mutant cell lacking LpsB glycotransferase have a highly truncated LPS glycoform containing only two carbohydrate residues bound to lipid A, resulting in decreased resistance to human serum and decreased survival in a rat model of soft tissue infection (Luke et al., 2010; McConnell et al., 2013). Inhibiting LpxC, an enzyme involved in the lipid A biosynthesis, dose not inhibit growth of the bacterium, but suppresses *A. baumannii* LPS-mediated activation of TLR4 (Lin et al., 2012). Inhibition of LpxC in mouse model enhances clearance of *A. baumannii* by enhancing opsonophagocytic killing and reduces serum LPS concentration and inflammation, which completely protects mice from lethal infection (Lin et al., 2012; Lee et al., 2013b). These results indicate that blocking LPS synthesis is a powerful strategy for discovering novel antibiotics. Modification of LPS contributes to resistance to antimicrobials. Many studies have shown that modifications in LPS decrease the susceptibility of *A. baumannii* to many clinical important antibiotics, such as colistin (Moffatt et al., 2010; Arroyo et al., 2011; Beceiro et al., 2011; Pelletier et al., 2013; Boll et al., 2015; Chin et al., 2015).

Phospholipase

Phospholipase is a lipolytic enzyme essential for phospholipid metabolism and is a virulence factor in many bacteria, such as *P. aeruginosa*, *Legionella monocytogenes*, and *Clostridium perfringens* (Camarena et al., 2010; Flores-Diaz et al., 2016). Three classes of phospholipases, such as phospholipase A (PLA), phospholipase C (PLC), and phospholipase D (PLD) have been defined based on the cleavage site. PLA hydrolyzes fatty acids from the glycerol backbone, whereas PLC cleaves the phosphorylated head group from the phospholipid. PLD is a transphosphatidylase that only cleaves off the head group. Degradation of phospholipids affects the stability of host cell membranes, and the cleaved head group can interfere with cellular signaling, resulting in changes in the host immune response (Songer, 1997; Flores-Diaz et al., 2016). PLC and PLD have been identified as virulence factors in *A. baumannii* (Camarena et al., 2010; Jacobs et al., 2010; Stahl et al., 2015). *Acinetobacter baumannii* ATCC17978 has two PLCs (A1S_0043 and A1S_2055) and inactivation of the A1S_0043 gene leads to a modest reduction in the cytotoxic effect of *A. baumannii* on epithelial cells compared to that of the parental strain (Camarena et al., 2010; Fiester et al., 2016). Disrupting one (A1S_2989) of the two PLD genes present in *A. baumannii* strain 98-37-09 results in reduced resistance to human serum, decreased capacity for invading epithelial cells, and decreased virulence in a murine model of pneumonia (Jacobs et al., 2010). Another report showed that *A. baumannii* ATCC 19606 has three PLD genes and all three play important roles in virulence and host cell invasion in a concerted manner (Stahl et al., 2015). These results suggest that phospholipase enzymes are important virulence factors in *A. baumannii* pathogenesis.

Outer Membrane Vesicles (OMVs)

OMVs are spherical, 20–200 nm diameter vesicles secreted by the outer membranes of various Gram-negative pathogenic bacteria (Kulp and Kuehn, 2010). They are composed of LPS, outer membrane and periplasmic proteins, phospholipids, and DNA or RNA, and are recognized as delivery vehicles for bacterial effectors to host cells (Ellis and Kuehn, 2010). OMVs deliver diverse virulence factors to the interior of host cells simultaneously and allow the pathogens to interact with the host without close contact between bacteria and host cells (Jun et al., 2013). Many *A. baumannii* strains secrete OMVs containing various virulence factors, including OmpA (Kwon et al., 2009; Jin et al., 2011; Moon et al., 2012), proteases (Kwon et al., 2009), and phospholipases (Kwon et al., 2009). OMVs derived from *A. baumannii* interact with host cells and deliver bacterial effectors to host cells via lipid rafts, resulting in cytotoxicity (Jin et al., 2011). Purified OMVs of *A. baumannii* ATCC 19606 induce expression of pro-inflammatory cytokine genes in epithelial cells in a dose-dependent manner (Jun et al., 2013). Notably, OMVs treated with proteinase do not induce a significant increase in the expression of pro-inflammatory cytokine genes, suggesting that the membrane proteins in OMVs are responsible for eliciting a potent innate immune response (Jun et al., 2013). One study supports the role of OMVs in *A. baumannii* pathogenesis. An *A. baumannii* strain that produces abundant OMVs with more virulence factors induces a stronger innate immune response and is more cytotoxic compared with those of a strain producing fewer OMVs (Li Z. T. et al., 2015).

Due to the importance of OMVs in *A. baumannii* virulence, several reports have shown that *A. baumannii* OMVs could be used as an acellular vaccine to elevate protective immunity (McConnell et al., 2011; Huang et al., 2014). In a mouse model of disseminated sepsis, vaccination with *A. baumannii* ATCC 19606 strain OMVs protects mice from challenge with homologous bacteria and provides protection against other clinical isolates (McConnell et al., 2011). Similar results were obtained in a pneumonia mouse model. Bacterial burden, inflammatory cell infiltration, and inflammatory cytokine accumulation in the pneumonia model were significantly suppressed by both active and passive immunization with OMVs (Huang et al., 2014). These results indicate that *A. baumannii* OMVs can be used as an acellular vaccine to effectively control *A. baumannii* infections. Interestingly, *A. baumannii* OMVs are also related with the spread of antibiotic resistance and induce the horizontal transfer of the OXA-24 carbapenemase gene (Rumbo et al., 2011).

Metal Acquisition System

Although iron is one of the most abundant elements in environmental and biological systems, ferric iron is relatively unavailable to bacteria in the preferred state, because of its poor solubility (10^{-17} M solubility limit for ferric iron) under aerobic and neutral pH conditions as well as due to chelation by low-molecular-weight compounds, such as heme, or high-affinity

iron-binding compounds, such as lactoferrin and transferrin (Rakin et al., 2012; Saha et al., 2013). To overcome this iron limitation, most aerobic bacteria produce a high-affinity iron chelator known as a siderophore (Saha et al., 2013). Siderophores are low molecular weight compounds (400–1,000 kDa) with high affinity for iron. The range of Fe^{3+}-siderophore association constants is 10^{12}–10^{52} (Saha et al., 2013). Siderophores have been classified into catecholates, hydroxymates, and a mixed type based on the moiety that donates oxygen ligands to coordinate Fe^{3+} (Saha et al., 2013). *Acinetobacter baumannii* also has iron siderophores and acinetobactin, the best-characterized *A. baumannii* siderophore, is a mixed type siderophore with an oxazoline ring derived from threonine (McConnell et al., 2013). Acinetobactin is an *A. baumannii* virulence factor (Gaddy et al., 2012; Penwell et al., 2012; Megeed et al., 2016). Impaired acinetobactin biosynthesis and transport functions significantly reduce the ability of *A. baumannii* ATCC 19606 cells to persist within epithelial cells and cause cell damage and animal death (Gaddy et al., 2012). Mutation in the *entA* gene, which is essential for biosynthesis of the acinetobactin precursor 2,3-dihydroxybenzoic acid, also significantly reduces the capacity of *A. baumannii* ATCC 19606 cells to persist within human alveolar epithelial cells and diminishes the ability to infect and kill *Galleria mellonella* larvae (Penwell et al., 2012). One study showed that acinetobactin production occurs significantly more frequently in MDR *A. baumannii* isolates than that in avirulent isolates (Megeed et al., 2016).

The *A. baumannii* NfuA Fe-S scaffold protein, that participates in the formation of Fe-S clusters and plays a role in cell responses to iron chelation and oxidative stress, has also been identified as a virulence factor (Zimbler et al., 2012). The *nfuA* mutant is more sensitive to reactive oxygen species (ROS), such as hydrogen peroxide and cumene hydroperoxide, and shows significantly reduced growth in human epithelial cells. In addition, a *G. mellonella* infection model showed that more than 50% of injected *G. mellonella* larvae die 6 days after infection with the parental strain, whereas less than 30% of the larvae die when infected with the *nfuA* mutant (Zimbler et al., 2012). One report showed that iron starvation increases production of PLCs, which increase hemolytic activity of *A. baumannii* (Fiester et al., 2016). These reports indicate that iron acquisition functions play a critical role in *A. baumannii* virulence.

The innate immune metal-chelating protein calprotectin inhibits bacterial growth by host-mediated chelation of metals, such as zinc (Zn^{2+} and Zn) and manganese (Mn^{2+} and Mn) (Corbin et al., 2008). However, *A. baumannii* can cause disease in the presence of this nutritional immune protein *in vivo* (Juttukonda et al., 2016). To combat the zinc limitation, *A. baumannii* uses a zinc acquisition system (ZnuABC), which is up-regulated under Zn-limiting conditions, and the *znuB* mutant strain experiences Zn starvation at higher Zn concentrations than that of the wild-type (Hood et al., 2012). ZnuB contributes to the pathogenesis of *A. baumannii* pulmonary infections. Notably, a zinc limitation reduces the imipenem MIC of MDR *A. baumannii* to below the clinical breakpoint for imipenem resistance in *A. baumannii* (Hood et al., 2012), possibly because many carbapenemases are metalloenzymes that require Zn for their hydrolyzing activity. Besides the ZnuABC system, the novel Zn metallochaperone ZigA has been characterized in *A. baumannii* (Nairn et al., 2016). ZigA tightly interacts with Zn, which is required for bacterial growth under Zn starvation conditions and for disseminated infection in mice (Nairn et al., 2016).

The mechanism employed by *A. baumannii* to overcome a Mn limitation has been identified. Calprotectin induces Mn starvation in *A. baumannii*, which increases transcription of an NRAMP (Natural Resistance-Associated Macrophage Proteins) family Mn transporter and a urea carboxylase to resist the antimicrobial activities of calprotectin (Juttukonda et al., 2016). A urea carboxylase enzyme (MumC) is important for growth of *A. baumannii* in the presence of calprotectin and an NRAMP family transporter (MumT) contributes to the fitness of *A. baumannii* in a murine pneumonia model (Juttukonda et al., 2016), suggesting that the two proteins are virulence factors. *Acinetobacter baumannii* can utilize urea as a sole nitrogen source, and this urea utilization is required for MumC (Juttukonda et al., 2016). Based on the contribution of MumC to *A. baumannii* resistance to calprotectin, the authors suggest a connection between metal starvation and metabolic stress, such as nitrogen starvation.

Protein Secretion Systems

Several protein secretion systems have been identified in *A. baumannii* (Weber et al., 2015a). The most recently described *A. baumannii* secretion system is a type II secretion system (T2SS) (Johnson et al., 2015). The T2SS is a multi-protein complex that is structurally very similar to type IV pili systems, which is an appendage that is commonly found in Gram-negative bacteria (Korotkov et al., 2012). T2SS translocates a wide range of proteins from the periplasmic space to the extracellular milieu out of the cell or the outer membrane surface. The T2SS is composed of 12–15 proteins comprised of four sub-assemblies: a pseudopilus, a cytoplasmic secretion ATPase, an inner-membrane platform assembly, and a dodecameric outer-membrane complex (Korotkov et al., 2012; Harding et al., 2016). Secretion by T2SS is a two-step process. The target proteins are first translocated to the periplasm by the general secretory (Sec) system or the twin arginine transport (Tat) system, where the target proteins are then secreted out of the cell through the T2SS (Korotkov et al., 2012). Deleting *A. baumannii* genes for the T2SS components, *gspD* or *gspE*, results in loss of LipA secretion, indicating that LipA is a T2SS substrate (Johnson et al., 2015). Because LipA is a lipase that breaks down long-chain fatty acids, *lipA*, *gspD*, and *gspE* mutant strains are incapable of growing on long-chain fatty acids as a sole carbon source and are defective in *in vivo* growth in a neutropenic murine model of bacteremia (Johnson et al., 2015). The role of a functional T2SS for full virulence of *A. baumannii* has been shown in *G. mellonella* and murine pulmonary infection models (Harding et al., 2016). Lipases (LipA, LipH, and LipAN) and the metallopeptidase CpaA have been identified as T2SS substrates (Elhosseiny et al., 2016; Harding et al., 2016). Notably, two proteins (LipA and CpaA) among these secreted proteins require specific chaperones for

secretion. These chaperones are encoded adjacently to their cognate effector, and their inactivation abolishes secretion of LipA and CpaA (Harding et al., 2016).

Acinetobacter baumannii also has a type VI secretion system (T6SS). The T6SS was first identified in *Vibrio cholera* and *P. aeruginosa* (Mougous et al., 2006; Pukatzki et al., 2006). Many bacteria use the T6SS to inject effector proteins, providing a colonization advantage during infection of eukaryotic hosts (Mougous et al., 2006) or to kill competing bacteria (Basler et al., 2013). The T6SS leads to DNA release and horizontal gene transfer in *V. cholera*, which may contribute to spread of antibiotic resistance (Borgeaud et al., 2015). The T6SS is composed of many conserved structural proteins and accessory factors, and bears a contractile bacteriophage sheath-like structure forming a needle or spike structure used to penetrate the target cell (Shneider et al., 2013). Hcp is a structural protein forming a polymerized tubular structure that is secreted out of the cell, and VgrGs are involved in attaching effector domains to the spike, and a proline-alanine-alanine-arginine (PAAR) repeat protein forms the sharp tip of the distinctive needle-like structure (Shneider et al., 2013; Zoued et al., 2014).

The presence of T6SS in *A. baumannii* was initially predicted by bioinformatic analysis (Weber et al., 2013). Although the role of T6SS in *A. baumannii* ATCC 17978 has not been determined (Weber et al., 2013), research on *A. baumannii* strain M2 showed that this strain produces a functional T6SS and that the T6SS mediates killing of competing bacteria (Carruthers et al., 2013). Another study showed that the T6SS is active in six pathogenic strains of *A. baumannii* (Ruiz et al., 2015). However, the T6SS seems to play an important role in *A. baumannii* virulence in a strain-specific manner (Repizo et al., 2015). They compared T6SS functionality of several *A. baumannii* strains, including ATCC17978 (a type strain), various MDR strains implicated in hospital outbreaks (Ab242, Ab244, and Ab825), and DSM30011 (a non-clinical isolate). Although the T6SS genomic locus is present in all of these strains, only DSM30011 has a fully active T6SS that mediates *E. coli* killing (Repizo et al., 2015). In addition, the T6SS of DSM30011 is required for host colonization of the *G. mellonella* model organism (Repizo et al., 2015). Similar results were obtained from a comparative analysis of the genomes of MDR *A. baumannii* clinical strains (Wright et al., 2014; Jones et al., 2015). *A. baumannii* isolates of a particular clade exhibit complete loss of the T6SS genomic locus. Therefore, these results suggest that more extensive investigations are required to analyze the role of T6SS in *A. baumannii* virulence, even though this system seems to play an important role in *A. baumannii* virulence in some strains. Notably, one study showed that several MDR *A. baumannii* strains have a large, self-transmissible plasmid that carries negative regulators for T6SS (Weber et al., 2015b). The T6SS is silenced in plasmid-containing, antibiotic-resistant cells, whereas plasmid-losing cells have an active T6SS. Although plasmid-losing cells are capable of T6SS-mediated killing of competing bacteria, they become susceptible to antibiotics (Weber et al., 2015b). This result suggests a molecular switch between T6SS and antibiotic resistance.

The type V system autotransporter Ata has also been characterized in *A. baumannii* (Bentancor et al., 2012a). This is a trimeric membrane protein that mediates biofilm formation, adherence to extracellular matrix components such as collagen I, III, and IV, and virulence in a murine systemic model of *Acinetobacter* infection (Bentancor et al., 2012a). Another experiment using a pneumonia model of infection in immunocompetent and immunocompromised mice showed that Ata is a vaccine candidate against *A. baumannii* infections (Bentancor et al., 2012b). A type IV secretion system present in the plasmid was bioinformatically identified in *A. baumannii* (Liu C. C. et al., 2014), but no experimental evidence describing its function has been presented.

Penicillin-Binding Protein 7/8 (PBP7/8) and β-Lactamase PER-1

Although PBPs are commonly involved in resistance to β-lactam antibiotics, PBP7/8 encoded by the *pbpG* gene is a virulence factor in *A. baumannii*. The *pbpG* mutant strain grows similar to its wild-type strain in Luria-Bertani medium, but the mutant shows reduced growth in human serum and its survival significantly decreases in rat soft-tissue infection and pneumonia models (Russo et al., 2009). An investigation of bacterial morphology using electron microscopy suggested that loss of PBP7/8 may have affected peptidoglycan structure, which may affect susceptibility to host defense factors (Russo et al., 2009).

Interestingly, β-lactamase PER-1 has been suggested to be an *A. baumannii* virulence factor. PER-1 is an extended-spectrum-β-lactamase (ESBL), but this gene is associated with cell adhesion (Sechi et al., 2004). Nine PER-1-producing strains adhere to the Caco2 cell lines, whereas all PER-1-negative strains are negative for cell adhesion (Sechi et al., 2004). Notably, many β-lactamases are associated with virulence in various pathogenic bacteria, such as *E. coli* (Dubois et al., 2009), *P. aeruginosa* (Moya et al., 2008), and *K. pneumoniae* (Sahly et al., 2008). However, no general mechanisms have been proposed (Beceiro et al., 2013).

Others

Acinetobacter baumannii CipA is a novel plasminogen binding and complement inhibitory protein that mediates serum resistance (Koenigs et al., 2016). CipA-binding plasminogen is converted to active plasmin that degrades fibrinogen and complement C3b, which contributes to serum resistance of *A. baumannii*. Therefore, the *cipA* mutant strain is efficiently killed by human serum and also shows a defect in the penetration of endothelial monolayers (Koenigs et al., 2016). Similar to CipA, the *A. baumannii* translation elongation factor Tuf is also a plasminogen-binding protein. Tuf-binding plasminogen can be converted to active plasmin, which proteolytically degrades fibrinogen as well as component C3b (Koenigs et al., 2015). RecA, which is involved in homologous recombination and the SOS response, has been identified as an *A. baumannii* virulence factor. The *recA* mutant shows significantly reduced survival within macrophages and decreases lethality in a mouse model of systemic infection (Aranda et al., 2011). The surface antigen protein 1 (SurA1) plays an important role in fitness and virulence of *A. baumannii* (Liu D. et al., 2016). Serum resistance of the *surA1* mutant significantly decreases compared with that of

the wild-type strain CCGGD201101. In the *G. mellonella* insect model, a *surA1* mutant strain exhibits a lower survival rate and decreased dissemination (Liu D. et al., 2016).

A growth analysis of 250,000 *A. baumannii* transposon mutants within *G. mellonella* larvae identified 300 genes required for survival or growth of *A. baumannii* inside *G. mellonella* larvae (Gebhardt et al., 2015). The 300 genes were classified into six categories of micronutrient acquisition, cysteine metabolism/sulfur assimilation, aromatic hydrocarbon metabolism, cell envelope/membrane/wall, stress response genes, antibiotic resistance, and transcriptional regulation. Among them, four transcriptional regulators required for growth in *G. mellonella* larvae were called the *gig* (growth in *Galleria*) genes. Loss of these genes (*gigA-D*) led to a significant defect in both growth within and killing of *G. mellonella* larvae (Gebhardt et al., 2015). This study identified stress proteins, such as UspA, as factors required for growth in *G. mellonella*. Another study showed that UspA is essential for pneumonia and sepsis pathogenesis of *A. baumannii* (Elhosseiny et al., 2015). Among the 300 genes, several genes are involved in aromatic hydrocarbon metabolism (Gebhardt et al., 2015). Another study showed that GacS, which is a transcriptional factor that regulates expression of genes, such as *paaE*, and is responsible for the phenylacetic acid catabolic pathway, affects *A. baumannii* virulence (Cerqueira et al., 2014). Experiments using a *paaE* deletion mutant confirmed the role of aromatic hydrocarbon metabolism in *A. baumannii* virulence (Cerqueira et al., 2014), but its molecular mechanism remains unknown. Interestingly, a recent report showed that accumulation of phenylacetate in *A. baumannii* induces rapid neutrophil influx to a localized site of infection and increases bacterial clearance (Bhuiyan et al., 2016). They suggested that phenylacetate is a neutrophil chemoattractant inducing bacterial-guided neutrophil chemotaxis. This report may reveal a novel molecular mechanism about the role of the phenylacetic acid catabolic pathway in *A. baumannii* virulence.

Biofilm formation plays an important role in immune evasion by *A. baumannii* (de Breij et al., 2010), and pili are essential for *A. baumannii* adherence to and biofilm formation on abiotic surfaces as well as virulence (Tomaras et al., 2003, 2008). Notably, imipenem treatment of the imipenem-resistant *A. baumannii* isolate induces expression of important genes responsible for synthesis of type IV pili (Dhabaan et al., 2015), suggesting that the ability to overproduce pili confers a biological advantage to *A. baumannii*.

Other virulence-related proteins have been identified, including OmpR/EnvZ (Tipton and Rather, 2016), FhaBC (Perez et al., 2016), and the resistance-nodulation-division-type membrane transporter AbeD (Srinivasan et al., 2015), but their molecular mechanisms remain unknown.

ANTIMICROBIAL RESISTANCE OF *A. BAUMANNII*

Acinetobacter baumannii has become one of the most successful pathogens in modern healthcare because of its amazing ability to acquire antimicrobial resistance. Several strains of *A. baumannii* are highly resistant to most clinically available antibiotics (Lin and Lan, 2014). *A. baumannii* has a number of resistance mechanisms, including β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, permeability defects, and modifications of target sites. The accumulation of several resistance mechanisms in *A. baumannii* has gradually decreased the number of antibiotic classes available to treat *A. baumannii* infections in clinical practice. **Table 2** shows the antibiotic resistance mechanisms found in *A. baumannii*. We will discuss the details below.

β-Lactamases

Inactivation of β-lactams by β-lactamases is a major antibiotic resistance mechanism in *A. baumannii*. Based on sequence homology, β-lactamases are grouped into molecular classes, A, B, C, and D (Jeon et al., 2015). All four classes of β-lactamases were identified in *A. baumannii*. Recent studies have shown that *A. baumannii* has natural competence to incorporate exogenous DNA and its genome has foreign DNA at high frequencies, implying frequent horizontal gene transfer in this pathogen (Ramirez et al., 2010a; Touchon et al., 2014; Traglia et al., 2014). Additionally, albumin, a main protein in blood, enhances natural competence of *A. baumannii* (Traglia et al., 2016). Therefore, natural competence of *A. baumannii* may contribute to identification of a large number of β-lactamases in this threatening human pathogen.

Class A β-lactamases inhibited by clavulanate hydrolyze penicillins and cephalosporins more efficiently than carbapenems, except for some KPC type enzymes (Jeon et al., 2015). A number of class A β-lactamases, including TEM, SHV, GES, CTX-M, SCO, PER, VEB, KPC, and CARB, have been identified in *A. baumannii* (**Table 2**). Some of these enzymes, such as TEM-1, CARB-4, and SCO-1, are narrow-spectrum β-lactamases, whereas other enzymes (e.g., PER-1, TEM-92, CARB-10, SHV-5, PER-2, CTX-M-2, CTX-M-15, VEB-1, GES-14, and PER-7) are ESBLs. Some carbapenemases, such as GES-14 and KPC-2, have been detected in *A. baumannii* (Moubareck et al., 2009; Bogaerts et al., 2010).

Unlike the serine-dependent β-lactamases (classes A, C, and D), class B β-lactamases are metallo-β-lactamases (MBLs) that require zinc or another heavy metal for catalysis (Jeon et al., 2015). Due to a broad substrate spectrum, MBLs catalyze the hydrolysis of virtually all β-lactam antibiotics including carbapenems, but not monobactams (Jeon et al., 2015). A variety of class B β-lactamases have been identified in *A. baumannii* (**Table 2**).

Class C β-lactamases pose therapeutic problems because they can confer resistance to cephamycins (cefoxitin and cefotetan), penicillins, cephalosporins, and β-lactamase inhibitor combinations, but are not significantly inhibited by clinically used β-lactamase inhibitors, such as clavulanic acid (Jeon et al., 2015). *Acinetobacter baumannii* has an intrinsic AmpC cephalosporinase (Gordon and Wareham, 2010). An analysis of 23 MDR *A. baumannii* clinical isolates in Taiwan showed that all isolates had AmpC-type β-lactamases (Lin et al., 2011a). Several clinical isolates of *A. baumannii* have the *ampC* gene transcribed

TABLE 2 | Resistance mechanisms in *Acinetobacter baumannii.*

Resistance mechanism	Class/subgroup	Protein	References
β-Lactamases	Class A	TEM-1	Chen et al., 2006; Adams et al., 2008; Krizova et al., 2013
		TEM-92	Endimiani et al., 2007
		GES-1	Al-Agamy et al., 2016
		GES-5	Al-Agamy et al., 2016
		GES-11	Moubareck et al., 2009; Bogaerts et al., 2010; Chihi et al., 2016
		GES-12	Bogaerts et al., 2010
		GES-14	Bogaerts et al., 2010
		PER-1	Jeong et al., 2005; Poirel et al., 2005a; Aly et al., 2016
		PER-2	Pasteran et al., 2006
		PER-7	Bonnin et al., 2011b
		CTX-M-2	Nagano et al., 2004
		CTX-M-15	Potron et al., 2011
		SCO-1	Poirel et al., 2007
		VEB-1	Fournier et al., 2006; Naas et al., 2006; Pasteran et al., 2006; Adams et al., 2008; Poirel et al., 2009
		KPC-2	Martinez et al., 2016
		KPC-10	Robledo et al., 2010
		CARB-4	Ramirez et al., 2010b
		CARB-10	Potron et al., 2009
	Class B	IMP-1	Tognim et al., 2006
		IMP-2	Riccio et al., 2000
		IMP-4	Chu et al., 2001
		IMP-5	Koh et al., 2007
		IMP-6	Gales et al., 2003
		IMP-8	Lee M. F. et al., 2008
		IMP-11	Yamamoto et al., 2011
		IMP-19	Yamamoto et al., 2011
		IMP-24	Lee M. F. et al., 2008
		VIM-1	Tsakris et al., 2006, 2008; Papa et al., 2009
		VIM-2	Yum et al., 2002; Lee M. F. et al., 2008
		VIM-3	Lee M. F. et al., 2008
		VIM-4	Tsakris et al., 2008; Papa et al., 2009
		VIM-11	Lee M. F. et al., 2008
		NDM-1	Chen et al., 2011; Pfeifer et al., 2011; Bonnin et al., 2012; Voulgari et al., 2016
		NDM-2	Espinal et al., 2011
		NDM-3	Kumar, 2016
		SIM-1	Lee et al., 2005
	Class C	AmpC	Bou and Martinez-Beltran, 2000; Corvec et al., 2003; Segal et al., 2004; Hujer et al., 2005; Heritier et al., 2006; Liu and Liu, 2015
	Class D		
	OXA-2 subgroup	OXA-21	Vila et al., 1997
	OXA-10 subgroup	OXA-128	Giannouli et al., 2009
	OXA-20 subgroup	OXA-37	Navia et al., 2002
	OXA-23 subgroup	OXA-23	Heritier et al., 2005b; Naas et al., 2005; Corvec et al., 2007; Koh et al., 2007; Perez et al., 2007; Valenzuela et al., 2007; Wang et al., 2007; Adams et al., 2008; Stoeva et al., 2008; Kohlenberg et al., 2009; Kuo et al., 2010; Mugnier et al., 2010; Bonnin et al., 2011a; Lee et al., 2011; Lin et al., 2011b; Koh et al., 2012; Mosqueda et al., 2013; Chagas et al., 2014; Principe et al., 2014; Li Y. et al., 2015

(Continued)

TABLE 2 | Continued

Resistance mechanism	Class/subgroup	Protein	References
		OXA-133	Mendes et al., 2009
		OXA-239	Gonzalez-Villoria et al., 2016
	OXA-24 subgroup	OXA-24	Bou et al., 2000b; Merino et al., 2010; Acosta et al., 2011; Pailhories et al., 2016
		OXA-25, OXA-26, OXA-27	Afzal-Shah et al., 2001
		OXA-40	Heritier et al., 2003; Lolans et al., 2006; Quinteira et al., 2007; Ruiz et al., 2007
		OXA-72	Wang et al., 2007; Lu et al., 2009; Goic-Barisic et al., 2011; Dortet et al., 2016; Kuo et al., 2016
		OXA-143	Higgins et al., 2009
		OXA-182	Kim et al., 2010
	OXA-51 subgroup	OXA-51	Brown et al., 2005; Hu et al., 2007; Ruiz et al., 2007; Adams et al., 2008; Chen et al., 2010; Fang et al., 2016
		OXA-64, OXA-65, OXA-66, OXA-68, OXA-70, OXA-71	Hamouda et al., 2010; Biglari et al., 2016
		OXA-69, OXA-75, OXA-76, OXA-77	Heritier et al., 2005a
		OXA-79, OXA-80, OXA-104, OXA-106∼ OXA-112	Evans et al., 2007
		OXA-82, OXA-83, OXA-83, OXA-84	Turton et al., 2006b; Evans et al., 2007
		OXA-86, OXA-87	Vahaboglu et al., 2006
		OXA-88, OXA-91, OXA-93, OXA-94, OXA-95, OXA-96	Koh et al., 2007
		OXA-92	Tsakris et al., 2007
		OXA-113	Naas et al., 2007
	OXA-58 subgroup	OXA-58	Dijkshoorn et al., 1996; Poirel et al., 2005b; Pournaras et al., 2006; Chen et al., 2008; Qi et al., 2008; Donnarumma et al., 2010; Gogou et al., 2011; Ravasi et al., 2011; Hou and Yang, 2015
		OXA-96	Koh et al., 2007
		OXA-97	Poirel et al., 2008
	OXA-143 subgroup	OXA-253	de Sa Cavalcanti et al., 2016
	OXA-235 subgroup	OXA-235	Higgins et al., 2013
Efflux pumps	Resistance-nodulation-division superfamily	AdeABC	Magnet et al., 2001; Marchand et al., 2004; Peleg et al., 2007; Ruzin et al., 2007; Lin et al., 2015; Sun et al., 2016
		AdeFGH	Coyne et al., 2010; He X. et al., 2015
		AdeIJK	Damier-Piolle et al., 2008
	Major facilitator superfamily	TetA	Ribera et al., 2003a
		TetB	Vilacoba et al., 2013
		CmlA	Coyne et al., 2011
		CraA	Roca et al., 2009
		AmvA	Rajamohan et al., 2010
		AbaF	Sharma et al., 2016
	Multidrug and toxic compound extrusion family	AbeM	Su et al., 2005
	Small multidrug resistance family	AbeS	Srinivasan et al., 2009
	Other efflux pumps	EmrAB-TolC	Nowak-Zaleska et al., 2016
		A1S_1535, A1S_2795, and ABAYE_0913	Li L. et al., 2016
Permeability defects	Porin	OmpA	Smani et al., 2014; Wu et al., 2016
		CarO	Mussi et al., 2005, 2007; Siroy et al., 2005; Catel-Ferreira et al., 2011; Jin et al., 2011

(Continued)

TABLE 2 | Continued

Resistance mechanism	Class/subgroup	Protein	References
		Omp22-33	Bou et al., 2000a
		Omp33-36	del Mar Tomas et al., 2005
		Omp37	Quale et al., 2003
		Omp43	Dupont et al., 2005
		Omp44	Quale et al., 2003
		Omp47	Quale et al., 2003
Aminoglycoside-modifying enzymes	Aminoglycoside acetyltransferases	AAC3 (*aacC1*, *aacC2*)	Nemec et al., 2004
		AAC(6′) (*aacA4*)	Doi et al., 2004; Cho et al., 2009; Zhu et al., 2009; Lin et al., 2010; Lin M. F. et al., 2013; Bakour et al., 2014
	Aminoglycoside adenyltransferases	ANT(2″) (*aadB*)	Nemec et al., 2004
		ANT(3″) (*aadA1*)	Cho et al., 2009; Lin et al., 2010; Lin M. F. et al., 2013
	Aminoglycoside phosphotransferases	APH(3′) (*aphA1*)	Gallego and Towner, 2001
		APH(3″)	Cho et al., 2009
Alteration of target sites	Change of penicillin binding protein(PBP)	PBP2	Gehrlein et al., 1991
	16S rRNA methylation	ArmA	Yu et al., 2007; Cho et al., 2009; Karthikeyan et al., 2010; Brigante et al., 2012; Hong et al., 2013; Bakour et al., 2014; Tada et al., 2014
	Ribosomal protection	TetM	Ribera et al., 2003b
	DNA gyrase	GyrA/ParC	Higgins et al., 2004
	Dihydrofolate reductase	DHFR	Mak et al., 2009; Lin M. F. et al., 2013
		FolA	Mak et al., 2009
	Lipopolysaccharide	PmrC, LpxA, LpxC, LpxD	Adams et al., 2009; Moffatt et al., 2010; Arroyo et al., 2011
Other mechanisms	S-adenosyl-L-methionine-dependent methyltransferase	Trm	Chen et al., 2014; Trebosc et al., 2016
	1-Acyl-sn-3-phosphate acyltransferase	PlsC	Li X. et al., 2015
	Peptidase C13 family	Abrp	Li X. et al., 2016
	Cell division proteins	BlhA, ZipA, ZapA, and FtsK	Knight et al., 2016
	SOS response	RecA	Aranda et al., 2011, 2014; Norton et al., 2013

from a strong promoter contained within a putative insertion sequence element (IS*Aba1*-like sequence), which results in high resistance to ceftazidime (Corvec et al., 2003; Segal et al., 2004). This sequence has been identified in ceftazidime-resistant *A. baumannii* isolates, but is absent in ceftazidime-susceptible *A. baumannii* isolates (Heritier et al., 2006).

Class D β-lactamases are called OXAs (oxacillinases), because they commonly hydrolyze isoxazolylpenicillin oxacillin much faster than benzylpenicillin (Jeon et al., 2015). More than 400 OXA-type enzymes have been identified and many variants actually possess carbapenemase activity. The presence of carbapenem-hydrolyzing class D β-lactamases or MBLs is one of the major carbapenem resistance mechanisms in *A. baumannii* (Lin and Lan, 2014). The subgroups of carbapenem-hydrolyzing OXAs, such as the OXA-23, OXA-24, OXA-51, and OXA-58 subgroups, are prevalent in *A. baumannii* (**Table 2**). The OXA-23 enzyme was first identified in an *A. baumannii* isolate in the United Kingdom in 1985 (Perez et al., 2007). The $bla_{\text{OXA-23}}$ gene has been disseminated worldwide, and the frequency of OXA-23-producing *A. baumannii* strains is significantly high (Mugnier et al., 2010; Al-Agamy et al., 2016). One recent report from Lebanon showed 76.5% of 119 *A. baumannii* isolates are resistant to carbapenems, and OXA-23 β-lactamases have been found in 82 isolates (Al Atrouni et al., 2016). Insertion of IS*Aba1* in the $bla_{\text{OXA-23}}$ promoter sequence has been reported to be associated with overexpression of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-51}}$, or $bla_{\text{OXA-58}}$ in *A. baumannii* (Turton et al., 2006a). One report from India showed that $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23}}$ were present in all 103 carbapenem-resistant *A. baumannii* isolates and almost 80% of the isolates had IS*Aba1* upstream of the $bla_{\text{OXA-23}}$ gene, indicating the prevalence of the IS*Aba1* insertion (Vijayakumar et al., 2016).

Efflux Pumps

Efflux pumps are associated with resistance against many different classes of antibiotics, such as imipenem (Hu et al., 2007) and tigecycline (Peleg et al., 2007; Ruzin et al., 2007), in *A. baumannii*. Reversal of antibiotic resistance by

efflux pump inhibitors, such as 1-(1-naphthylmethyl)-piperazine and carbonyl cyanide 3-chlorophenyl-hydrazone, supports the importance of efflux pumps in *A. baumannii* antibiotic resistance (Pannek et al., 2006; Deng et al., 2014). Four categories of efflux pumps, such as the resistance-nodulation-division superfamily, the multidrug and toxic compound extrusion family, the major facilitator superfamily, and the small multidrug resistance family transporters, are related to antimicrobial resistance in *A. baumannii* (**Table 2**; Lin and Lan, 2014).

AdeABC in the resistance-nodulation-division superfamily is associated with aminoglycoside resistance (Magnet et al., 2001) and with decreasing susceptibility to tigecycline (Ruzin et al., 2007) and non-fluoroquinolone antibiotics (Higgins et al., 2004). AdeABC seems to be cryptic in wild-type *A. baumannii* because of stringent control by the AdeRS two-component system (Marchand et al., 2004), but point mutations or insertion of the IS*Aba1* sequence in the *adeS* gene leads to overexpression of AdeABC (Marchand et al., 2004; Sun et al., 2012, 2016; Hammerstrom et al., 2015). Cell density (Fernando and Kumar, 2012) and the BaeSR two-component system (Lin et al., 2014, 2015), which is involved in an envelope stress response, also seem to regulate transcription of the *adeA* gene and thus affect tigecycline susceptibility. Other resistance-nodulation-division type efflux pumps, such as AdeFGH and AdeIJK, are synergistically associated with tigecycline resistance (Damier-Piolle et al., 2008). AdeFGH and AdeIJK expression is regulated by the LysR-type transcriptional regulator AdeL and the TetR-type transcriptional regulator AdeN (Coyne et al., 2010; Rosenfeld et al., 2012).

Acinetobacter baumannii clinical isolates possess a strong ability to form biofilms (Rodriguez-Bano et al., 2008). Notably, the subinhibitory concentrations of antibiotics encountered by low-dose therapy seem to strongly induce biofilm formation (Kaplan, 2011). A recent result revealed the mechanism. Overexpression of the AdeFGH efflux pump by low-dose antimicrobial therapy increases the synthesis and transport of autoinducer molecules, which induce biofilm formation (He X. et al., 2015). These results suggest a link between low-dose antimicrobial therapy and a high risk for biofilm infections caused by *A. baumannii*.

CmlA and CraA are major facilitator superfamily efflux pumps related with chloramphenicol (Fournier et al., 2006; Roca et al., 2009), and TetA is associated with tetracycline resistance (Ribera et al., 2003a). The novel efflux pump AmvA mediates resistance to different classes of antibiotics, disinfectants, detergents, and dyes, such as erythromycin, acriflavine, benzalkonium chloride, and methyl viologen (Rajamohan et al., 2010). AbaF was recently identified as a novel efflux pump associated with fosfomycin resistance (Sharma et al., 2016).

AbeM is in the multidrug and toxic compound extrusion family and confers resistance to imipenem and fluoroquinolones (Su et al., 2005). AbeS is the small multidrug resistance family transporter and affects resistance to various antimicrobial compounds. Deletion of the *abeS* gene results in increased susceptibility to various antimicrobial compounds, such as chloramphenicol, nalidixic acid, and erythromycin (Srinivasan et al., 2009).

Some other efflux pumps, such as MacAB-TolC (Kobayashi et al., 2001) and EmrAB-TolC (Lomovskaya and Lewis, 1992), have been well described in *E. coli*, but their role in *A. baumannii* has been recently explored. The EmrAB-TolC efflux pump is also present in *A. baumannii* where it conferred resistance to netilmicin, tobramycin, and imipenem (Nowak-Zaleska et al., 2016). Another report identified three novel efflux pumps (A1S_1535, A1S_2795, and ABAYE_0913) in *A. baumannii* using multiplexed phenotypic screening (Li L. et al., 2016). A1S_1535 confers resistance to various antibiotics, including gentamicin, kanamycin, chloroxylenol, oxytetracycline, 1,10-phenanthroline, and chloramphenicol (Li L. et al., 2016). A1S_2795 is the first major facilitator superfamily efflux pump found to confer resistance to the sulphonamide sulfathiazole, and ABAYE_0913 is associated with resistance to chloramphenicol and fusidic acid (Li L. et al., 2016).

Permeability Defects

A change in envelope permeability can influence antibiotic resistance. For example, porins form channels that allow transport of molecules across the outer membrane and play a significant role in *A. baumannii* virulence (**Table 1**). Because porins affect membrane permeability, they also play a significant role in the mechanism of resistance. Reduced expression of some porins, including CarO (Mussi et al., 2005, 2007; Siroy et al., 2005; Catel-Ferreira et al., 2011; Jin et al., 2011), Omp22-33 (Bou et al., 2000a), Omp33-36 (del Mar Tomas et al., 2005; Hood et al., 2010), Omp37 (Quale et al., 2003), Omp43 (Dupont et al., 2005), Omp44 (Quale et al., 2003), and Omp47 (Quale et al., 2003), is associated with carbapenem resistance in *A. baumannii*. Loss of Omp29 in *A. baumannii* producing OXA-51-like or OXA-23-like carbapenemases results in increased imipenem resistance (Jeong et al., 2009; Fonseca et al., 2013). OmpA is also related with resistance to aztreonam, chloramphenicol, and nalidixic acid (Smani et al., 2014). One study showed that OmpA and CarO physically interact with OXA-23 carbapenemase, and these interactions are associated with antibiotic resistance (Wu et al., 2016). These results provide a novel view to increase understanding of bacterial antibiotic resistance mechanisms.

Besides outer membrane proteins, envelope components, such as LPS and peptidoglycans, also affects antibiotic resistance of *A. baumannii*. Loss or modification of LPS decreases membrane integrity and increases colistin resistance in *A. baumannii* (Adams et al., 2009; Moffatt et al., 2010).

Aminoglycoside-Modifying Enzymes

Aminoglycoside-modifying enzymes are the major mechanism by which *A. baumannii* confers resistance to aminoglycosides. Aminoglycoside-modifying enzymes can be classified into acetyltransferases, adenyltransferases, and phosphotransferases. These enzymes are typically present on transposable elements and are transferred among pathogenic bacteria (Lin and Lan, 2014). Several reports show that many MDR *A. baumannii* isolates produce a combination of aminoglycoside-modifying enzymes (Gallego and Towner, 2001; Nemec et al., 2004). A study from China identified a MDR *A. baumannii* strain carrying four aminoglycoside-modifying enzymes (Zhu et al., 2009). Another

study from Greece reported that all *A. baumannii* strains contain aminoglycoside-modifying enzymes (Ploy et al., 1994), indicating the high prevalence of these enzymes in *A. baumannii*.

Alteration of Target Sites

Modifications in antibiotic target sites for antibiotics can induce antibiotic resistance in *A. baumannii*. In the absence of other known resistance mechanisms, only overexpression of altered PBPs with a low affinity for imipenem induce imipenem resistance (Gehrlein et al., 1991). Quinolone resistance is associated with modifications in GyrA (one subunit of DNA gyrase) and ParC (one subunit of topoisomerase IV) in epidemiologically unrelated *A. baumannii* isolates (Vila et al., 1995). *Acinetobacter baumannii* TetM, which has 100% homology with *S. aureus* TetM, has been proposed to be associated with tetracycline resistance through ribosomal protection (Ribera et al., 2003b). Similar to other pathogenic bacteria, dihydrofolate reductases (DHFR and FolA) responsible for trimethoprim resistance have been found in nosocomial MDR *A. baumannii* isolates (Mak et al., 2009; Lin M. F. et al., 2013; Taitt et al., 2014). The 16S rRNA methylase ArmA responsible for aminoglycoside resistance is also found in many *A. baumannii* strains and always coexists with OXA type carbapenemases such as OXA-23 (Yu et al., 2007; Cho et al., 2009; Karthikeyan et al., 2010; Brigante et al., 2012; Hong et al., 2013; Bakour et al., 2014; Tada et al., 2014; Hasani et al., 2016). As described above, many studies have shown that modifications or/and loss of LPS decrease the susceptibility of *A. baumannii* to many clinical important antibiotics, such as colistin.

Others

AdeABC is associated with decreased susceptibility to tigecycline (Ruzin et al., 2007). However, some clinical isolates without overexpressed AdeABC, AdeFGH, and AdeIJK have decreased susceptibility to tigecycline. Several reports have suggested the mechanism. One study analyzed eight *A. baumannii* clinical isolates and revealed that the deletion mutation in the *trm* gene, which encodes S-adenosyl-L-methionine-dependent methyltransferase, decreases susceptibility to tigecycline (Chen et al., 2014). The same result was reported using a highly efficient and versatile genome-editing platform enabling markerless modification of the *A. baumannii* genome. Deletion of AdeR, a transcription factor that regulates AdeABC efflux pump expression in tigecycline-resistant *A. baumannii*, reduces the MIC of tigecycline. However, 60% of the clinical isolates remained nonsusceptible to tigecycline after the *adeR* deletion according to a highly efficient and versatile genome-editing platform (Trebosc et al., 2016). Whole-genome sequencing in two tigecycline-resistant *adeR* deletion strains revealed that a mutation in the *trm* gene makes the *adeR* mutant resistant to tigecycline. In addition, a *trm* disruption was identified in most tigecycline-resistant clinical isolates (Trebosc et al., 2016). However, its exact mechanism was not determined. Another study revealed that a frameshift mutation in *plsC*, encoding 1-acyl-*sn*-glycerol-3-phosphate acyltransferase, is associated with decreased susceptibility to tigecycline (Li X. et al., 2015).

The *abrp* gene, which encodes the peptidase C13 family, is associated with decreased susceptibility to tetracycline, minocycline, doxycycline, tigecycline, chloramphenicol, and fosfomycin (Li X. et al., 2016). Deletion of *abrp* increases cell membrane permeability, displays slower cell growth rate, and confers reduced susceptibility to these antibiotics (Liu X. et al., 2016). However, its exact mechanism was not determined. Some genes involved in cell division, including *blhA*, *zipA*, *zapA*, and *ftsK*, are associated with intrinsic β-lactam resistance in *A. baumannii* (Knight et al., 2016).

Increased expression of mutagenesis-related genes, such as the SOS response genes, is a well-understood mechanism of *E. coli* and other bacteria to obtain antibiotic resistance (Cirz and Romesberg, 2007). *Acinetobacter baumannii* also seems to have an inducible DNA damage response in which RecA plays a major regulatory role and seems to acquire antibiotic resistances under clinically relevant DNA-damaging conditions (Aranda et al., 2011, 2014; Norton et al., 2013). Furthermore, RecA is involved in the *A. baumannii* pathogenicity (Aranda et al., 2011).

PROSPECTIVE TREATMENT OPTIONS

Although carbapenems are effective antibiotics to treat *A. baumannii* infections (Cisneros and Rodríguez-Baño, 2002; Turner et al., 2003), the rate of carbapenem-resistant *A. baumannii* isolates has been increasing gradually (Mendes et al., 2010; Kuo et al., 2012; Su et al., 2012). Only a few effective antibiotic options are available to treat MDR *A. baumannii* infections (Gordon and Wareham, 2009; Lee J. H. et al., 2015, 2016). To combat MDR or pandrug-resistant (PDR) *A. baumannii*, which are resistant to all available antibiotics, combination therapies, including colistin/imipenem, colistin/meropenem, colistin/rifampicin, colistin/tigecycline, colistin/sulbactam, colistin/teicoplanin, and imipenem/sulbactam, have been extensively studied. Prospective treatment options of *Acinetobacter baumannii* infections are summarized in **Table 3**. We will discuss the most recent published reports.

Carbapenems and β-Lactamase Inhibitors

Carbapenems, including imipenem, meropenem, and doripenem, have generally been considered the agents to treat *A. baumannii* infections, due to their effective activity against this organism and their favorable safety (Doi et al., 2015). However, the decreased susceptibility of *A. baumannii* to carbapenems has forced clinicians and researchers to explore alternative therapeutic approaches (Doi et al., 2015). Because carbapenem-resistant *A. baumannii* strains are often resistant to all other commonly used antibiotics as well, these strains remain susceptible to only limited antibiotics, such as minocycline/tigecycline and polymyxins (colistin and polymyxin B; Lin and Lan, 2014; Doi et al., 2015). Carbapenem therapies combined with a few effective antibiotics was extensively tested and many cases showed a synergistic effect against *A. baumannii* infections (**Table 3**). However, recent increase of tigecycline- or colistin-resistant *A. baumannii* increasingly poses a serious

TABLE 3 | Prospective treatment options of *Acinetobacter baumannii* infections.

Drugs	Type of research	Type of *A. baumannii*	Findings	References
Carbapenem +ampicillin+sulbactam+	*In vivo*	Carbapenem-resistant	Combination therapy with ampicillin-sulbactam and meropenem is effective against skin and soft tissue infection	Hiraki et al., 2013
	In vivo	Multidrug-resistant	The combination of a carbapenem and ampicillin/sulbactam was associated with a better outcome than the combination of a carbapenem and amikacin, or a carbapenem alone	Kuo et al., 2007
Carbapenem +minocycline	*In vitro*	Multidrug-resistant	Minocycline in combination with rifampicin, imipenem, and colistin showed bactericidal synergy in most of the isolates which did not harbor the *tetB* gene, but the combinations were not synergistic in *tetB*-positive isolates	Rodriguez et al., 2015
Carbapenem +tigecycline+colistin	Case report	Multidrug-resistant, colistin-susceptible	A patient with bacteremia had a favorable clinical outcome by a meropenem/colistin/tigecycline combination therapy	Candel et al., 2010
Carbapenem +colistin	*In vitro*/case report	Extensively drug-resistant, colistin-susceptible	Effective; 80% of patients were treated successfully	Ozbek and Senturk, 2010
	In vitro	Multidrug-resistant, colistin-susceptible	Imipenem/colistin showed best synergy effects	Pongpech et al., 2010
	In vitro/case report	Multidrug-resistant, colistin-susceptible	Meropenem/colistin can inhibit bacterial regrowth at 24 h	Lee C. H. et al., 2008
	In vitro	Colistin-susceptible and colistin-resistant	Subinhibitory meropenem/colistin showed synergy against 49 of 52 strains at 24 h	Pankuch et al., 2008
	In vitro	Extensively drug-resistant, colistin-susceptible	Combinations of colistin/rifampicin, colistin/meropenem, colistin/minocycline and minocycline/meropenem are synergistic	Liang et al., 2011
	A retrospective study	Extensively drug-resistant, colistin-susceptible	Colistin/carbapenem and colistin/sulbactam resulted in significantly higher microbiological eradication rates, relatively higher cure and 14-day survival rates, and lower in-hospital mortality compared to colistin monotherapy in patients with bloodstream infections	Batirel et al., 2014
	In vitro	Carbapenem-resistant, colistin-susceptible	Synergistic effects against all 12 isolates	Liu X. et al., 2016
	In vivo	Extensively drug-resistant, colistin-susceptible and colistin-resistant	Colistin/fusidic acid and colistin/rifampicin were synergistic in a murine thigh-infection model; The colistin-meropenem combination was also effective when the colistin MIC is ≤32 mg/L.	Fan et al., 2016
	In vitro	Extensively drug-resistant	The daptomycin-colistin combination was the most effective; the colistin/imipenem combination was also effective	Cordoba et al., 2015
Carbapenem +colistin+rifampicin	Case report	Multidrug-resistant, colistin-susceptible	Successful treatment by a meropenem/colistin/rifampicin combination therapy in a case of multifocal infection	Biancofiore et al., 2007
Carbapenem+plazomicin	*In vitro*	Carbapenem-resistant	Synergistic activity	Garcia-Salguero et al., 2015
Imipenem+polymyxin B	*In vitro*	Carbapenem-resistant	Doripenem, meropenem, or imipenem displayed similar pharmacodynamics in combination with polymyxin B	Lenhard et al., 2016b
Meropenem+ polymyxin B	*In vitro*	Multidrug-resistant	Combinations of polymyxin B/meropenem and polymyxin B/meropenem/fosfomycin showed high synergistic activity	Menegucci et al., 2016

(Continued)

TABLE 3 | Continued

Drugs	Type of research	Type of *A. baumannii*	Findings	References
	In vitro/in vivo	Carbapenem-resistant	Intensified meropenem dosing in combination with polymyxin B synergistically killed carbapenem-resistant strains, irrespective of the meropenem MIC	Lenhard et al., 2016a
	In vitro	Carbapenem-resistant	Doripenem, meropenem, or imipenem displayed similar pharmacodynamics in combination with polymyxin B	Lenhard et al., 2016b
Doripenem+tigecycline	*In vitro*	Multidrug-resistant, doripenem-resistant	Synergistic activity	Principe et al., 2013
Doripenem+colistin	*In vitro*	Multidrug-resistant, doripenem-resistant	Synergistic activity	Principe et al., 2013
Doripenem+polymyxin B	*In vitro*	Carbapenem-resistant	Doripenem, meropenem, or imipenem displayed similar pharmacodynamics in combination with polymyxin B	Lenhard et al., 2016b
	In vitro	Polymyxin-heteroresistant	The polymyxin B/doripenem combination resulted in rapid and extensive initial killing within 24 h, which was sustained over 10 days	Rao et al., 2016a
Doripenem+amikacin	*In vitro*	Multidrug-resistant, doripenem-resistant	Synergistic activity	Principe et al., 2013
Ampicillin+sulbactam	*In vitro/in vivo*	Multi-drug resistant	Ampicillin/sulbactam therapy significantly decreased the risk of death in patients with bloodstream infections	Smolyakov et al., 2003
Sulbactam+colistin	A retrospective study	Extensively drug-resistant, colistin-susceptible	Colistin/carbapenem and colistin/sulbactam resulted in significantly higher microbiological eradication rates, relatively higher cure and 14-day survival rates, and lower in-hospital mortality compared to colistin monotherapy in patients with bloodstream infections	Batirel et al., 2014
	A retrospective study	Multidrug-resistant	The colistin/sulbactam combination therapy is promising in patients with ventilator-associated pneumonia	Kalin et al., 2014
Tazobactam+colistin	*In vivo*	Colistin-susceptible	Tazobactam plus colistin showed synergy	Sakoulas et al., 2016
Minocycline+colistin	*In vitro*	Extensively drug-resistant	Combinations of colistin/rifampicin, colistin/meropenem, colistin/minocycline and minocycline/meropenem are synergistic	Liang et al., 2011
	In vitro/in vivo	Minocycline-resistant	Minocycline/colistin synergistically killed minocycline-resistant isolates; minocycline/colistin also significantly improved the survival of mice and reduced the number of bacteria present in the lungs of mice	Yang et al., 2016
	In vitro	Multidrug-resistant	Minocycline in combination with rifampicin, imipenem, and colistin showed bactericidal synergy in most of the isolates which did not harbor the *tetB* gene, but the combinations were not synergistic in *tetB*-positive isolates	Rodriguez et al., 2015
Minocycline+rifampicin	*In vivo*	Multidrug-resistant	Synergistic effect of minocycline/rifampicin and minocycline/amikacin combinations in a mouse lung infection model	He S. et al., 2015
	In vitro	Multidrug-resistant	Minocycline in combination with rifampicin, imipenem, and colistin showed bactericidal synergy in most of the isolates which did not harbor the *tetB* gene, but the combinations were not synergistic in *tetB*-positive isolates	Rodriguez et al., 2015

(Continued)

TABLE 3 | Continued

Drugs	Type of research	Type of *A. baumannii*	Findings	References
Minocycline+amikacin	*In vivo*	Multidrug-resistant	Synergistic effect of minocycline/rifampicin and minocycline/amikacin combinations in a mouse lung infection model	He S. et al., 2015
Tigecycline+colistin	*In vitro*	Carbapenem-resistant, colistin-susceptible	Good synergy	Ozbek and Senturk, 2010; Sheng et al., 2011
	In vitro	Extensively drug-resistant, colistin-susceptible	Good synergy	Dizbay et al., 2010
	In vitro	Tigecycline-non-susceptible	Good synergy	Principe et al., 2009
	In vitro	Carbapenem-resistant, colistin-susceptible and colistin-resistant	Good synergy	Peck et al., 2012
	In vitro/in vivo	Extensively drug-resistant	*In vitro* synergistic activity; no statistically significant differences were found between colistin, tigecycline, and combination treatments in terms of efficacy on bacterial counts in lung tissue of a rat pneumonia model	Mutlu Yilmaz et al., 2012
Tigecycline+polymyxin B	*In vitro*	Carbapenem-resistant, polymyxin-heteroresistant	Combination of polymyxin B-with higher tigecycline concentrations result in sustained bactericidal activity	Rao et al., 2016b
	In vitro	Carbapenem-resistant	Synergistic effects in combination therapy with simulated exposures of polymyxin B and tigecycline at an aggressive dose	Hagihara et al., 2014
Tigecycline+amikacin	*In vitro*	Multidrug-resistant	Synergistic bactericidal activities	Moland et al., 2008
Colistin+rifampicin	*In vitro/in vivo*	Multidrug-resistant, colistin-susceptible	Efficacy *in vitro* and in experimental models of pneumonia and meningitis	Pachon-Ibanez et al., 2010
	Case report	Carbapenem-resistant, colistin-susceptible	Efficacy in 7 of 10 patients with ventilator-associated pneumonia	Song et al., 2008
	Case report	Multidrug-resistant, colistin-susceptible	Efficacy in 22 of 29 critically ill patients with pneumonia and bacteremia	Bassetti et al., 2008
	In vivo	Multidrug-resistant, colistin-susceptible	Synergistic effect in prolonging survival	Pantopoulou et al., 2007
	Clinical trial	Multidrug-resistant, colistin-susceptible	Favorable for all 26 nosocomial infection patients	Motaouakkil et al., 2006
	In vitro	Carbapenem-resistant, colistin-susceptible	Effective for strains highly resistant to imipenem and moderately resistant to rifampicin	Montero et al., 2004
	In vitro	Multidrug-resistant, colistin-susceptible	Synergistic effect against 11 of 13 isolates	Hogg et al., 1998
	In vitro	Extensively drug-resistant	Combinations of colistin/rifampicin, colistin/meropenem, colistin/minocycline and minocycline/meropenem are synergistic	Liang et al., 2011
	In vitro	Multidrug-resistant, colistin-susceptible	Colistin/rifampicin was fully synergistic against 4 of 5 isolates; colistin/meropenem and colistin/azithromycin were synergistic against 3 of 5 isolates; colistin/doxycycline was partially synergistic or additive against 5 isolates	Timurkaynak et al., 2006
	Case report	Carbapenem-resistant, colistin-susceptible	Rifampicin/colistin and ampicillin/sulbactam resulted in microbiological clearance in 9 of 14 critically ill patients	Petrosillo et al., 2005
	In vitro	Carbapenem-resistant, colistin-heteroresistant	Rifampicin/colistin and imipenem/colistin were synergistic against heteroresistant isolates and prevented the development of colistin-resistant strains	Rodriguez et al., 2010
	Case report	Carbapenem-resistant, colistin-susceptible	Synergistic effect in patients with ventilator-associated pneumonia	Aydemir et al., 2013

(Continued)

TABLE 3 | Continued

Drugs	Type of research	Type of *A. baumannii*	Findings	References
	In vivo	Extensively drug-resistant, colistin-susceptible and colistin-resistant	Colistin/fusidic acid and colistin/rifampicin were synergistic in a murine thigh-infection model; The colistin-meropenem combination was also effective when the colistin MIC is ≤32 mg/L.	Fan et al., 2016
	In vitro	Colistin-resistant	The most effective combinations were colistin-rifampin and colistin-teicoplanin; both combinations showed synergistic effect against 8 of 9 colistin-resistant strains	Bae et al., 2016
Colistin+teicoplanin	*In vitro/in vivo*	Multidrug-resistant, colistin-susceptible	Synergistic effect of colistin/daptomycin and colistin/teicoplanin in a mouse model	Cirioni et al., 2016
	In vitro	Multidrug-resistant, colistin-susceptible	Significant synergy	Wareham et al., 2011
	In vitro	Colistin-resistant	The most effective combinations were colistin-rifampin and colistin-teicoplanin; both combinations showed synergistic effect against 8 of 9 colistin-resistant strains	Bae et al., 2016
Colistin+daptomycin	*In vitro/in vivo*	Multidrug-resistant, colistin-susceptible	Synergistic effect of colistin/daptomycin and colistin/teicoplanin in a mouse model	Cirioni et al., 2016
	In vitro	Extensively drug-resistant	The daptomycin-colistin combination was the most effective; the colistin/imipenem combination was also effective	Cordoba et al., 2015
Colistin+vancomycin	*In vitro/in vivo*	Multidrug-resistant, colistin-susceptible	Highly active both in vitro and in an animal model of *Galleria mellonella*	Hornsey and Wareham, 2011
Colistin+fosfomycin	*In vitro*	Carbapenem-resistant, colistin-susceptible	Good synergy; no synergy between colistin and sulbactam, colistin and imipenem	Santimaleeworagun et al., 2011
Colistin+fusidic acid	*In vitro*	Carbapenem-resistant, colistin-susceptible and colistin-resistant	*In vitro* synergy between colistin and fusidic acid that is comparable to the synergy between colistin and vancomycin; the synergy with fusidic acid is strain-dependent and applicable to strains for which the colistin MICs are relatively low	Bowler et al., 2016
	In vitro	Carbapenem-resistant, colistin-susceptible and colistin-resistant	Robust synergy between fusidic acid and colistin against multidrug-resistant clinical strains, including some colistin-resistant strains	Phee et al., 2015
	In vivo	Extensively drug-resistant, colistin-susceptible and colistin-resistant	Colistin/fusidic acid and colistin/rifampicin were synergistic in a murine thigh-infection model; The colistin-meropenem combination was also effective when the colistin MIC is ≤32 mg/L.	Fan et al., 2016
Colistin+amikacin	Case report	Multidrug-resistant, colistin-susceptible	Successful clinical and microbiological outcomes	Fulnecky et al., 2005
Colistin+trimethoprim-sulfamethoxazole	*In vitro*	Carbapenem-resistant	Colistin/trimethoprim-sulfamethoxazole killed effectively all carbapenem-resistant strains	Nepka et al., 2016
Polymyxin B+netropsin	*In vitro/in vivo*	Colistin-resistant	The survival of infected *Galleria mellonella* was significantly higher when treated with polymyxin B and netropsin in combination than when treated with polymyxin B or netropsin alone	Chung et al., 2016

(Continued)

TABLE 3 | Continued

Drugs	Type of research	Type of *A. baumannii*	Findings	References
Trimethoprim-sulfamethoxazole	*In vitro*	Carbapenem-resistant	Trimethoprim-sulfamethoxazole killed effectively all carbapenem-resistant strains	Nepka et al., 2016
Novobiocin	*In vitro*	Carbapenem-susceptible	Inhibition of frequency of the occurrence of rifampin resistance mutants	Jara et al., 2015
Bacteriophages	*In vitro/in vivo*	Carbapenem-resistant, carbapenem-susceptible	Strong lytic activities and the improvement of survival rates	Jeon et al., 2016; Kusradze et al., 2016
Endolysin (LysABP-01)+colistin	*In vitro*	Multidrug-resistant	Synergistic activity	Thummeepak et al., 2016
Artilysins	*In vitro*	Carbapenem-resistant, carbapenem-susceptible	Artilysins are effective *in vitro* and *in vivo*	Briers et al., 2014; Yang et al., 2015; Defraine et al., 2016; Thandar et al., 2016
Antimicrobial peptides	*In vitro*	Multidrug-resistant	Good antimicrobial activities	Pires et al., 2015; Barksdale et al., 2016
Rose bengal+ carbapenem	*In vitro*	Carbapenem-resistant	Imipenem or meropenem with rose bengal showed synergistic effects	Chiu et al., 2016
β-Aminoketone (MD3)+colistin	*In vitro*	Colistin-susceptible, colistin-resistant	Synergistic effect targeting to strains with specific colistin resistance mechanisms; synergy against both colistin-susceptible strains and colistin-resistant strains with mutations in *pmrB* and phosphoethanolamine modification of lipid A, but not against colistin-resistant strains with loss of lipopolysaccharide	Martinez-Guitian et al., 2016
Bulgecin A+ carbapenem	*In vitro*	Carbapenem-resistant	Synergistic activity	Skalweit and Li, 2016
Farnesol+colistin	*In vitro*	Colistin-resistant	Farnesol increased sensitivity to colistin for colistin-resistant strains	Kostoulias et al., 2015
Oleanolic acid+gentamicin or kanamycin	*In vitro*	Carbapenem-susceptible	Synergistic activity	Shin and Park, 2015
Cyanide 3-chlorophenylhydrazone (CCCP)+colistin	*In vitro*	Colistin-resistant	CCCP reversed colistin resistance and inhibited the regrowth of the resistant subpopulation	Ni et al., 2016
	In vitro	Colistin-resistant	Synergistic activity	Park and Ko, 2015
ABEPI1 or ABEPI2+minocycline	*In vitro*	Carbapenem-susceptible	Synergistic activity	Blanchard et al., 2014
Gallium nitrate	*In vitro/in vivo*	Multidrug-resistant	Good antimicrobial activities; protection of *Galleria mellonella* larvae from lethal *A. baumannii* infection; synergistic activity with colistin	Antunes et al., 2012
Gallium protoporphyrin IX	*In vitro/in vivo*	Multidrug-resistant	Good antimicrobial activities	Arivett et al., 2015
Gallium nitrate+colistin	*In vitro/in vivo*	Multidrug-resistant	Good antimicrobial activities; protection of *Galleria mellonella* larvae from lethal *A. baumannii* infection; synergistic activity with colistin	Antunes et al., 2012

(Continued)

TABLE 3 | Continued

Drugs	Type of research	Type of *A. baumannii*	Findings	References
D-amino acids	*In vitro/in vivo*	Carbapenem-susceptible	Some D-amino acids (D-histidine and D-cysteine) can inhibit bacterial growth, biofilm formation and adherence to eukaryotic cells	Rumbo et al., 2016
Bifidobacterium breve strain Yakult	*In vivo*	Multidrug-resistant	Protection against fatal intestinal infection in a murine infection model	Asahara et al., 2016
Clarithromycin	*In vivo*	Multidrug-resistant	Inhibition of bacterial growth and biofilm formation; immunomodulator	Konstantinidis et al., 2016
Lysophosphatidylcholine +carbapenem	*In vivo*	Multidrug-resistant strain	Lysophosphatidylcholine in combination with colistin, tigecycline, or imipenem markedly enhanced the bacterial clearance from the spleen and lungs and reduced bacteremia and mouse mortality rates	Parra Millan et al., 2016
Lysophosphatidylcholine +tigecycline	*In vivo*	Multidrug-resistant strain	Lysophosphatidylcholine in combination with colistin, tigecycline, or imipenem markedly enhanced the bacterial clearance from the spleen and lungs and reduced bacteremia and mouse mortality rates	Parra Millan et al., 2016
Lysophosphatidylcholine +colistin	*In vivo*	Multidrug-resistant strain	Lysophosphatidylcholine in combination with colistin, tigecycline, or imipenem markedly enhanced the bacterial clearance from the spleen and lungs and reduced bacteremia and mouse mortality rates	Parra Millan et al., 2016

threat to public health worldwide (Peleg et al., 2007; Hornsey et al., 2010; Cai et al., 2012).

Sulbactam is a β-lactamase inhibitor and also has affinity for penicillin-binding proteins of *A. baumannii* (Rafailidis et al., 2007; Doi et al., 2015). Combined therapy of ampicillin with sulbactam is effective for treating bloodstream infections due to MDR *A. baumannii* (Smolyakov et al., 2003). Ampicillin/sulbactam/carbapenem combination therapy is also effective for treating MDR *A. baumannii* bacteremia (Kuo et al., 2007) and skin and soft tissue infection of carbapenem-resistant *A. baumannii* (Hiraki et al., 2013), but not in ventilator-associated pneumonia (Kalin et al., 2014). The population pharmacokinetics and pharmacodynamics of sulbactam were determined in critically ill patients with severe sepsis caused by *A. baumannii* (Jaruratanasirikul et al., 2016) and in patients with impaired renal function (Yokoyama et al., 2015). Another β-lactamase inhibitor tazobactam increases the activity of peptide antibiotics, such as colistin and daptomycin, in a murine model of *A. baumannii* pneumonia (Sakoulas et al., 2016). The authors suggested that β-lactamase inhibitors may exert similar effects in potentiating peptide antibiotics, because of structural similarities between β-lactamase inhibitors and peptide antibiotics (Sakoulas et al., 2016).

Minocycline/Tigecycline

Minocycline, is a broad-spectrum tetracycline antibiotic that has been proposed for treating drug-resistant *A. baumannii* based on its high degree of susceptibility to this drug and its favorable pharmacokinetic profile (Ritchie and Garavaglia-Wilson, 2014). The mean susceptibility rate of *A. baumannii* to minocycline is approximately 80% worldwide (Castanheira et al., 2014). Therefore, minocycline therapy has high treatment success rates and good tolerability (Ritchie and Garavaglia-Wilson, 2014). However, since the introduction of minocycline, approximately 20% of *A. baumannii* isolates are not susceptible to minocycline. The TetB efflux pump is the main determinants of minocycline resistance (Vilacoba et al., 2013). Minocycline therapy combined with colistin is effective for treating minocycline-resistant *A. baumannii* infections (Yang et al., 2016), and minocycline therapy combined with rifampicin, colistin, or imipenem has a synergistic effect in most of isolates without the *tetB* gene, but combined therapies are not synergistic in isolates with the *tetB* gene (Rodriguez et al., 2015).

Tigecycline is the first glycylcycline class antibiotic that exhibits bacteriostatic activity by binding to the 30S ribosomal subunit, and is active against *A. baumannii* infections (Pachon-Ibanez et al., 2004; Anthony et al., 2008; Koomanachai et al., 2009). Tigecycline shows a synergistic effect with some classes of antibiotics, such as amikacin (Moland et al., 2008) and colistin (Mutlu Yilmaz et al., 2012). However, limitations of tigecycline use have emerged with its increasing use. Tigecycline is less effective than imipenem to treat pneumonia in a murine pneumonia model (Pichardo et al., 2010). β-Lactam or carbapenem instead of tigecycline was recommended for *A. baumannii* infections with tigecycline MIC of more than 2 mg/L, due to high mortality from the tigecycline treatment (Curcio and Fernandez, 2008). In a study of 266 patients with MDR

A. baumannii infections, tigecycline-based therapy was not more effective than non-tigecycline-based therapies (Lee Y. T. et al., 2013). Tigecycline resistance associated with overexpression of efflux pumps, such as AdeABC, has been reported in clinical isolates of *A. baumannii* (Peleg et al., 2007; Ruzin et al., 2007; Hornsey et al., 2010, 2011). Multiple MDR *A baumannii* clones resistant to tigecycline have been reported in many medical centers (Navon-Venezia et al., 2007). Therefore, tigecycline can only be used in limited cases for treating *A. baumannii* infections.

Polymyxins (Colistin and Polymyxin B)

Polymyxins are a group of polycationic peptide antibiotics that were discovered more than 60 years ago and exhibit potent efficacy against most Gram-negative bacteria (Liu Q. et al., 2014; Lee C. R. et al., 2016). Among all five polymyxins (A–E), only polymyxin B and E (colistin) with a one amino acid difference are used clinically. Colistin is a key component of combination therapies used to treat MDR *A. baumannii* infections (Cai et al., 2012). The rate of colistin resistance (10.4%) in MDR *A. baumannii* isolates is lower than that of rifampicin (47.8%) or tigecycline (45.5%) resistance (Chang et al., 2012). Similar results were reported in another study (Muthusamy et al., 2016). Therefore, colistin seems to be the only effective antimicrobial agent against MDR *A. baumannii* infections. Many colistin-based combined therapies, including colistin/rifampicin (Liang et al., 2011; Aydemir et al., 2013), colistin/minocycline (Liang et al., 2011), colistin/carbapenem (Liang et al., 2011; Batirel et al., 2014; Liu X. et al., 2016), colistin/sulbactam (Batirel et al., 2014), colistin/tigecycline (Principe et al., 2009; Ozbek and Senturk, 2010; Sheng et al., 2011; Peck et al., 2012), colistin/daptomycin (Cirioni et al., 2016), colistin/fusidic acid (Bowler et al., 2016; Fan et al., 2016), and colistin/teicoplanin (Wareham et al., 2011; Cirioni et al., 2016), are synergistic *in vivo* or *in vitro* against *A. baumannii* infections. Colistin therapy combined with rifampin or fusidic acid seems to be the most effective for treating a MDR *A. baumannii* in a murine thigh-infection model (Fan et al., 2016). Another report comparing colistin/daptomycin, colistin/imipenem, and imipenem/ertapenem showed that the daptomycin-colistin combination was the most effective (Cordoba et al., 2015).

Unfortunately, the emergence of colistin-resistant *A. baumannii* strains has increased worldwide (Cai et al., 2012). The mechanisms of colistin resistance include loss of LPS (Moffatt et al., 2010) and the addition of phosphoethanolamine to LPS by the PmrAB two-component system (Adams et al., 2009). Mutations in *pmrA* and *pmrB* activate *pmrC*, which adds phosphoethanolamine to the hepta-acylated form of lipid A (Beceiro et al., 2011). Interestingly, an investigation of the *in vitro* activities of various antimicrobial combinations against colistin-resistant *A. baumannii* showed that the most effective combinations against colistin-resistant *A. baumannii* are colistin-rifampin and colistin-teicoplanin, indicating that colistin is the most common constituent of antimicrobial combinations even against colistin-resistant *A. baumannii* (Bae et al., 2016). Similarly, minocycline therapy in combination with colistin is effective to treat infections caused by minocycline-resistant *A. baumannii*. Minocycline/colistin therapy significantly improves survival of mice infected with minocycline-resistant *A. baumannii* and reduces the number of bacteria present in the lungs of mice (Yang et al., 2016).

A urinary tract *Enterococcus faecalis* isolate that apparently requires vancomycin to grow was reported in 1994, and this phenomenon is called "antimicrobial agent dependence." Colistin dependence was reported in an *A. baumannii–A. calcoaceticus* complex (Hawley et al., 2007). Partial colistin dependence has been detected in several LPS-deficient strains with mutations in *lpxA*, *lpxC*, and *lpxD* (Garcia-Quintanilla et al., 2015). Many colistin-susceptible *A. baumannii* isolates develop colistin dependence *in vitro* after exposure to colistin (Hong et al., 2016). Although the clinical implication of colistin dependence and its molecular mechanism remain unclear, it is interesting that patients with colistin-dependent *A. baumannii* isolates show a high rate of treatment failure (Hong et al., 2016).

Unlike colistin, polymyxin B is not converted from a prodrug form into an active form; thus, plasma concentrations of polymyxin B more quickly reach target levels (Sandri et al., 2013). In addition, polymyxin B is available for direct parenteral administration (Zavascki et al., 2007). Despite the favorable pharmacokinetics of polymyxin B, dose-related nephrotoxicity limits the concentration of polymyxin B used in combination therapy (Dubrovskaya et al., 2015). Therefore, almost all studies on polymyxins are carried out for colistin. However, because some carbapenems have comparatively safer dose modulation to optimize killing during combination therapy (Cannon et al., 2014), several studies have analyzing the pharmacodynamics of carbapenems in combination with polymyxin B (Lenhard et al., 2016a,b; Rao et al., 2016a). One study showed that intensified meropenem dosing combined with polymyxin B is a good strategy to treat carbapenem-resistant *A. baumannii*, regardless of the meropenem MIC (Lenhard et al., 2016a). Combination therapy with doripenem and polymyxin B also showed similar results. Early aggressive dosing of doripenem combined with polymyxin B is effective for treating heteroresistant *A. baumannii* infections (Rao et al., 2016a). A combined pharmacodynamics analysis of four different carbapenems with polymyxin B showed that doripenem, meropenem, or imipenem display similar pharmacodynamics in combination, and the decision to use carbapenem in combination with polymyxin B is usually based on toxicodynamic profiles (Lenhard et al., 2016b). Polymyxin B also shows good bactericidal activity in combination with high tigecycline concentrations (Hagihara et al., 2014; Rao et al., 2016b). Therefore, polymyxin B combination therapies seem to be one of the most promising options for minimizing the emergence of polymyxin resistance. Increasing the dose intensity of polymyxin B amplifies polymyxin B resistance in *A. baumannii* (Cheah et al., 2016; Tsuji et al., 2016). In conclusion, although polymyxin B displays dose-related nephrotoxicity, it is a potential therapeutic alternative to colistin when use together with intensified doses of other antibiotics. Large-scale screening of *Streptomyces* secondary metabolites was performed to develop a novel combination therapy using minimal concentrations of polymyxin B, and the reliable polymyxin synergist netropsin was identified (Chung et al., 2016). Survival of *G. mellonella*

infected with colistin-resistant clinical *A. baumannii* isolates is significantly higher when treated with polymyxin B combined with netropsin than when treated with polymyxin B or netropsin alone (Chung et al., 2016).

Other Antibiotics

Trimethoprim-sulfamethoxazole is a two antibiotics combination that exerts a synergistic effect by inhibiting successive steps in the folate synthesis pathway against a number of bacteria (Wormser et al., 1982). The *in vitro* killing activity of trimethoprim-sulfamethoxazole against carbapenem-resistant *A. baumannii* was recently studied. Trimethoprim-sulfamethoxazole alone effectively kills all carbapenem-resistant *A. baumannii* strains and trimethoprim-sulfamethoxazole combined with colistin also rapidly kills all strains for up to 24 h (Nepka et al., 2016). These results suggest that trimethoprim-sulfamethoxazole might be an effective therapy for severe carbapenem-resistant *A. baumannii* infections. Plazomicin is a next-generation aminoglycoside synthetically derived from sisomicin that enhances activity against many MDR Gram-negative bacteria (Garcia-Salguero et al., 2015). A synergistic effect was observed with carbapenems along with plazomicin during treatment of *A. baumannii* infections (Garcia-Salguero et al., 2015), indicating the potential utility of plazomicin combined with carbapenems.

The inducible DNA damage response in *A. baumannii* plays an important role in acquiring antibiotic resistance under clinically relevant DNA-damaging conditions (Aranda et al., 2011, 2014; Norton et al., 2013). The aminocoumarin novobiocin is a well-established antimicrobial agent that inhibits the DNA damage response in Gram-positive bacteria by interfering with ATPase activity of DNA gyrase (Schroder et al., 2013). One study showed that novobiocin also inhibits acquisition of antimicrobial resistance in MDR *A. baumannii* through DNA damage-induced mutagenesis (Jara et al., 2015).

Non-antibiotic Therapies: Phage and Others

The worldwide spread of MDR pathogens has renewed interest in the therapy using bacteriophage, which is a virus that infects and lyses bacteria. Various lytic *A. baumannii* bacteriophages, such as *vB_Ab-M-G7* (Kusradze et al., 2016) and Bϕ-C62 (Jeon et al., 2016), have been used to treat infections caused by MDR *A. baumannii*. Bacteriophage-encoded endolysin has also received attention. Endolysin is a lytic enzyme that degrades the cell wall of bacterial hosts and shows promise as a novel class of antibacterials with a unique mode of action (Defraine et al., 2016). For example, endolysin from *A. baumannii* bacteriophage ØABP-01 degrades the crude cell wall of *A. baumannii* strains and elevates antibacterial activity when combined with colistin (Thummeepak et al., 2016). However, most Gram-negative pathogens are generally not susceptible to endolysins, due to their protective outer membrane (Lee et al., 2013a). To overcome this problem, endolysins have recently been engineered with specific outer membrane-destabilizing peptides to obtain the ability to penetrate outer membrane and these engineered endolysins are called "artilysins" (Rodriguez-Rubio et al., 2016). Several engineered artilysins have been developed to combat MDR *A. baumannii* and show highly effective antimicrobial activity against *A. baumannii* (Briers et al., 2014; Yang et al., 2015; Defraine et al., 2016; Thandar et al., 2016). These results suggest that artilysins can be a treatment option for MDR *A. baumannii*. The diversity of the phage population was determined by analysis of viromes, endolysins, and CRISPR spacers (Davison et al., 2016). These results can be used to assist in finding an effective endolysin for combating MDR *A. baumannii*. Various peptides, such as American alligator plasma peptide (Barksdale et al., 2016) and antimicrobial peptide dendrimer G3KL (Pires et al., 2015), have *in vitro* antimicrobial activity against MDR *A. baumannii*. However, the use of antimicrobial enzymes or peptides also has some important problems, such as their short half-life in serum and high production costs compared with those of smaller molecules.

An *in silico* analysis predicted that OXA-58, OXA-23, and OXA-83 are translocated to the periplasm via the Sec system (Liao et al., 2015; Chiu et al., 2016). A SecA inhibitor (rose bengal) inhibits periplasmic translocation of these carbapenem-hydrolyzing class D β-lactamases, indicating that these β-lactamases are selectively released via a Sec-dependent system (Liao et al., 2015; Chiu et al., 2016). Imipenem or meropenem combined with rose bengal shows synergistic effects for carbapenem-resistant *A. baumannii* clinical isolates (Chiu et al., 2016). Similarly, β-aminoketone (MD3), an inhibitor of bacterial type I signal peptidases that cleaves the amino-terminal signal peptides of translocated proteins, shows a synergistic effect when combined with colistin against colistin-resistant *A. baumannii* strains (Martinez-Guitian et al., 2016).

Bulgecin A is a natural product of *P. mesoacidophila* and a lytic transglycosylase inhibitor that works synergistically with β-lactams (Skalweit and Li, 2016). Bulgecin A restores the efficacy of meropenem in suppressing growth of carbapenem-resistant *A. baumannii* strains, suggesting that Bulgecin A may be an adjunctive compound to extend the life of carbapenems against *A. baumannii* infections (Skalweit and Li, 2016). Similarly, farnesol, a natural product of *Candida albicans* for quorum-sensing, disrupts *A. baumannii* cell membrane integrity, alters cell morphology, and increases sensitivity of MDR *A. baumannii* strains to colistin (Kostoulias et al., 2015). Many herbal active compounds have potent antibacterial activities against many bacteria including carbapenem-resistant *A. baumannii* (Lin et al., 2015). For example, oleanolic acid is a triterpenoid compound that widely exists in food, medicinal herbs, and many plants and can potently inhibit various pathogenic bacteria. One study showed that oleanolic acid increases aminoglycoside uptake by changing membrane permeability and energy metabolism in *A. baumannii* (Shin and Park, 2015).

Cyanide 3-chlorophenylhydrazone (CCCP) is an efflux pump inhibitor that decreases the MIC of colistin in colistin-susceptible and colistin-resistant *A. baumannii* strains (Park and Ko, 2015; Ni et al., 2016). Other efflux pump inhibitors, such as ABEPI1 and ABEPI2, inhibit efflux-mediated minocycline tolerance of

A. baumannii. Adding these compounds during growth in human serum leads to the accumulation of minocycline within *A. baumannii* and inhibits efflux potential of the bacterium (Blanchard et al., 2014).

Gallium is a semi-metallic element in group 13 of the periodic table that binds to biological complexes containing Fe^{3+} and disrupts essential redox-driven biological processes (Bernstein, 1998). Gallium has been used as a simple inorganic or organic salt or complexed with organic compounds. Several studies have shown that gallium nitrate or gallium protoporphyrin IX could be a viable therapeutic option for treating MDR *A. baumannii* (Antunes et al., 2012; Arivett et al., 2015). Some D-amino acids, such as D-His and D-Cys, inhibit bacterial growth, biofilm formation, and adherence to eukaryotic cells in *A. baumannii* (Rumbo et al., 2016).

Probiotics are "live microorganisms that confer a health benefit on the host when administered in adequate amounts" (Reid et al., 2005) and assist in protecting against MDR *A. baumannii* infections. For example, the ability of the probiotic *Bifidobacterium breve* to protect against MDR *A. baumannii* infections has been investigated (Asahara et al., 2016). This probiotic markedly potentiates protection against fatal intestinal infections caused by MDR *A. baumannii* (Asahara et al., 2016). With probiotics, immunomodulators, such as lysophosphatidylcholine (Parra Millan et al., 2016) and macrolide antibiotics such as clarithromycin (Konstantinidis et al., 2016), can reduce *A. baumannii* infection severity by stimulating the immune response, when combined with antibiotics such as colistin, tigecycline, or imipenem.

CONCLUSION

The number of studies about *A. baumannii* is increasing dramatically because of its increasing clinical importance. Use of animal models has produced important data regarding virulence factors that contribute to *A. baumannii* pathogenesis. Notably, some studies on metal acquisition and protein secretion systems are interesting. Besides iron acquisition systems such as acinetobactin, the discovery of zinc and manganese acquisition systems in *A. baumannii* broadens our understanding of *A. baumannii* pathogenesis. More extensive studies on various protein secretion systems present in *A. baumannii* are required. About 300 genes required for *in vivo* survival of *A. baumannii* were identified using transposon screening in *G. mellonella* larvae (Gebhardt et al., 2015). Because many of these genes were not known to be associated with *A. baumannii* pathogenesis, more detailed studies are required to determine whether these genes are related to the pathogenesis of *A. baumannii*. In addition, transposon screening in other model animals will provide novel insight into *A. baumannii* pathogenesis. Knowledge of virulence factors responsible for *A. baumannii* pathogenicity will be the cornerstone for developing novel antibiotics. For example, LPS is an important virulence factor and LpxC inhibitor, which inhibits LPS synthesis, completely protects mice from lethal infection (Lin et al., 2012). These results indicate that blocking LPS synthesis is a powerful strategy for discovering novel antibiotics. However, despite recent extensive studies about *A. baumannii* pathogenesis, the toxicity and pathogenicity of *A. baumannii* remain unclear.

Recent interest about *A. baumannii* is mostly due to its seemingly endless capacity to acquire antibiotic resistance. *A. baumannii* has almost all bacterial resistance mechanisms. All class β-lactamases have been detected in *A. baumannii* and the frequency of carbapenem-resistant *A. baumannii* isolates is very high. Furthermore, almost all *A. baumannii* contain aminoglycoside-modifying enzymes and many efflux pumps responsible for resistance to various clinically important antibiotics have been identified in *A. baumannii*. Due to these abilities, available antibiotics to treat *A. baumannii* infections are significantly limited. Colistin is used as the antibiotic treatment of last resort, due to its relatively low resistance rate. However, emergence of colistin-resistant *A. baumannii* strains has increased worldwide with increasing use of colistin. Notably, some more recent studies have proposed that another polymyxin antibiotic, polymyxin B, is a potential therapeutic alternative to colistin (Lenhard et al., 2016a,b; Rao et al., 2016a; Repizo et al., 2015). Polymyxin B has not been a good antibiotic owing to dose-dependent nephrotoxicity, but recent reports show that a novel combination therapy with carbapenems or tigecycline using minimal concentrations of polymyxin B can be a good strategy to treat carbapenem-resistant *A. baumannii* infections. These results indicate the requirement for extensive studies that analyze the pharmacodynamics of polymyxin B in combination therapy.

Various trials to identify a novel alternative to carbapenem or colistin have been performed. Among them, engineered endolysins (artilysins) are particularly interesting, despite evident defects. A lytic enzyme degrading peptidoglycan of bacteria is a promising novel class of antimicrobial agents due to its unique mode of action. Similar to β-lactam antibiotics that are one of the most successful antibiotics, inhibition of peptidoglycan synthesis is a promising target of antimicrobial agents. Because lytic enzymes directly degrade peptidoglycans, but not proteins, the possibility of the emergence of a resistance mechanism is relatively low. In addition, enzymes with relatively high molecular weight are not inhibited by efflux pumps. If the short stability of artilysin in serum and high cost in its production compared with small molecules can be resolved, the improved artilysin can be a good treatment option for carbapenem- or colistin-resistant *A. baumannii* infections. In conclusion, novel, rationally designed strategies and screening-based approaches are required to discover new classes of antibiotics. If we continue to take all efforts at maintaining the effectiveness of antibiotics and developing novel antibiotics, effective control of *A. baumannii* infections can be successful.

AUTHOR CONTRIBUTIONS

CL, JL, MP, and SL contributed to the conception and the design of the review and CL, JL, MP, KP, IB, YK, CC, BJ, and SL researched and wrote the review.

FUNDING

This review was supported by the Cooperative Research Program for Agriculture Science and Technology Development (No. PJ01103103) of Rural Development Administration in Republic of Korea; the Environmental Health Action Program (No. 2016001350004) funded by the Ministry of Environment (MOE) in Republic of Korea; and the National Research Foundation of the Ministry of Education, Republic of Korea (NRF-2015R1C1A1A02037470).

REFERENCES

Acosta, J., Merino, M., Viedma, E., Poza, M., Sanz, F., Otero, J. R., et al. (2011). Multidrug-resistant *Acinetobacter baumannii* Harboring OXA-24 carbapenemase, Spain. *Emerg. Infect Dis.* 17, 1064–1067. doi: 10.3201/eid/1706.091866

Adams, M. D., Goglin, K., Molyneaux, N., Hujer, K. M., Lavender, H., Jamison, J. J., et al. (2008). Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J. Bacteriol.* 190, 8053–8064. doi: 10.1128/JB.00834-08

Adams, M. D., Nickel, G. C., Bajaksouzian, S., Lavender, H., Murthy, A. R., Jacobs, M. R., et al. (2009). Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob. Agents Chemother.* 53, 3628–3634. doi: 10.1128/AAC.00284-09

Afzal-Shah, M., Woodford, N., and Livermore, D. M. (2001). Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 45, 583–588. doi: 10.1128/AAC.45.2.583-588.2001. [Epub ahead of print].

Al-Agamy, M. H., Jeannot, K., El-Mahdy, T. S., Shibl, A. M., Kattan, W., Plesiat, P., et al. (2016). First Detection of GES-5 Carbapenemase-Producing *Acinetobacter baumannii* Isolate. *Microb. Drug Resist.* doi: 10.1089/mdr.2016.0152. [Epub ahead of print].

Al Atrouni, A., Hamze, M., Jisr, T., Lemarie, C., Eveillard, M., Joly-Guillou, M. L., et al. (2016). Wide spread of OXA-23-producing carbapenem-resistant *Acinetobacter baumannii* belonging to clonal complex II in different hospitals in Lebanon. *Int. J. Infect. Dis.* 52, 29–36. doi: 10.1016/j.ijid.2016.09.017

Aly, M. M., Abu Alsoud, N. M., Elrobh, M. S., Al Johani, S. M., and Balkhy, H. H. (2016). High prevalence of the PER-1 gene among carbapenem-resistant *Acinetobacter baumannii* in Riyadh, Saudi Arabia. *Eur. J. Clin. Microbiol. Infect Dis.* 35, 1759–1766. doi: 10.1007/s10096-016-2723-8

Anthony, K. B., Fishman, N. O., Linkin, D. R., Gasink, L. B., Edelstein, P. H., and Lautenbach, E. (2008). Clinical and microbiological outcomes of serious infections with multidrug-resistant gram-negative organisms treated with tigecycline. *Clin. Infect. Dis.* 46, 567–570. doi: 10.1086/526775

Antunes, L. C., Imperi, F., Carattoli, A., and Visca, P. (2011). Deciphering the multifactorial nature of *Acinetobacter baumannii* pathogenicity. *PLoS ONE* 6:e22674. doi: 10.1371/journal.pone.0022674

Antunes, L. C., Imperi, F., Minandri, F., and Visca, P. (2012). *In vitro* and *in vivo* antimicrobial activities of gallium nitrate against multidrug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 56, 5961–5970. doi: 10.1128/AAC.01519-12

Aranda, J., Bardina, C., Beceiro, A., Rumbo, S., Cabral, M. P., Barbe, J., et al. (2011). *Acinetobacter baumannii* RecA protein in repair of DNA damage, antimicrobial resistance, general stress response, and virulence. *J. Bacteriol.* 193, 3740–3747. doi: 10.1128/JB.00389-11

Aranda, J., Lopez, M., Leiva, E., Magan, A., Adler, B., Bou, G., et al. (2014). Role of *Acinetobacter baumannii* UmuD homologs in antibiotic resistance acquired through DNA damage-induced mutagenesis. *Antimicrob. Agents Chemother.* 58, 1771–1773. doi: 10.1128/AAC.02346-13

Arivett, B. A., Fiester, S. E., Ohneck, E. J., Penwell, W. F., Kaufman, C. M., Relich, R. F., et al. (2015). Antimicrobial activity of Gallium Protoporphyrin IX against *Acinetobacter baumannii* strains displaying different antibiotic resistance phenotypes. *Antimicrob. Agents Chemother.* 59, 7657–7665. doi: 10.1128/AAC.01472-15

Arroyo, L. A., Herrera, C. M., Fernandez, L., Hankins, J. V., Trent, M. S., and Hancock, R. E. (2011). The *pmrCAB* operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrob. Agents Chemother.* 55, 3743–3751. doi: 10.1128/AAC.00256-11

Asahara, T., Takahashi, A., Yuki, N., Kaji, R., Takahashi, T., and Nomoto, K. (2016). Protective effect of a synbiotic against multidrug-resistant *Acinetobacter baumannii* in a Murine Infection Model. *Antimicrob. Agents Chemother.* 60, 3041–3050. doi: 10.1128/AAC.02928-15

Aydemir, H., Akduman, D., Piskin, N., Comert, F., Horuz, E., Terzi, A., et al. (2013). Colistin vs. the combination of colistin and rifampicin for the treatment of carbapenem-resistant *Acinetobacter baumannii* ventilator-associated pneumonia. *Epidemiol. Infect.* 141, 1214–1222. doi: 10.1017/S095026881200194X

Bae, S., Kim, M. C., Park, S. J., Kim, H. S., Sung, H., Kim, M. N., et al. (2016). *In vitro* synergistic activity of antimicrobial agents in combination against clinical isolates of colistin-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 6774–6779. doi: 10.1128/AAC.00839-16

Bakour, S., Alsharapy, S. A., Touati, A., and Rolain, J. M. (2014). Characterization of *Acinetobacter baumannii* clinical isolates carrying bla_{OXA-23}carbapenemase and 16S rRNA methylase armA genes in Yemen. *Microb. Drug Resist.* 20, 604–609. doi: 10.1089/mdr.2014.0018

Barksdale, S. M., Hrifko, E. J., Chung, E. M., and van Hoek, M. L. (2016). Peptides from American alligator plasma are antimicrobial against multi-drug resistant bacterial pathogens including *Acinetobacter baumannii*. *BMC Microbiol.* 16:189. doi: 10.1186/s12866-016-0799-z

Basler, M., Ho, B. T., and Mekalanos, J. J. (2013). Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell* 152, 884–894. doi: 10.1016/j.cell.2013.01.042

Bassetti, M., Repetto, E., Righi, E., Boni, S., Diverio, M., Molinari, M. P., et al. (2008). Colistin and rifampicin in the treatment of multidrug-resistant *Acinetobacter baumannii* infections. *J. Antimicrob. Chemother.* 61, 417–420. doi: 10.1093/jac/dkm509

Batirel, A., Balkan, I. I., Karabay, O., Agalar, C., Akalin, S., Alici, O., et al. (2014). Comparison of colistin-carbapenem, colistin-sulbactam, and colistin plus other antibacterial agents for the treatment of extremely drug-resistant *Acinetobacter baumannii* bloodstream infections. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 1311–1322. doi: 10.1007/s10096-014-2070-6

Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J. A., Doumith, M., Hornsey, M., et al. (2011). Phosphoethanolamine modification of lipid A in colistin-resistant variants of Acinetobacter baumannii mediated by the *pmrAB* two-component regulatory system. *Antimicrob. Agents Chemother.* 55, 3370–3379. doi: 10.1128/AAC.00079-11

Beceiro, A., Tomas, M., and Bou, G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin. Microbiol. Rev.* 26, 185–230. doi: 10.1128/CMR.00059-12

Bentancor, L. V., Camacho-Peiro, A., Bozkurt-Guzel, C., Pier, G. B., and Maira-Litran, T. (2012a). Identification of Ata, a multifunctional trimeric autotransporter of *Acinetobacter baumannii*. *J. Bacteriol.* 194, 3950–3960. doi: 10.1128/JB.06769-11

Bentancor, L. V., Routray, A., Bozkurt-Guzel, C., Camacho-Peiro, A., Pier, G. B., and Maira-Litran, T. (2012b). Evaluation of the trimeric autotransporter Ata as a vaccine candidate against *Acinetobacter baumannii* infections. *Infect. Immun.* 80, 3381–3388. doi: 10.1128/IAI.06096-11

Bergogne-Berezin, E., and Towner, K. J. (1996). *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin. Microbiol Rev.* 9, 148–165.

Bernstein, L. R. (1998). Mechanisms of therapeutic activity for gallium. *Pharmacol. Rev.* 50, 665–682.

Bhuiyan, M. S., Ellett, F., Murray, G. L., Kostoulias, X., Cerqueira, G. M., Schulze, K. E., et al. (2016). *Acinetobacter baumannii* phenylacetic acid metabolism influences infection outcome through a direct effect on neutrophil chemotaxis. *Proc. Natl. Acad. Sci. U.S.A.* 113, 9599–9604. doi: 10.1073/pnas.1523116113

Biancofiore, G., Tascini, C., Bisa, M., Gemignani, G., Bindi, M. L., Leonildi, A., et al. (2007). Colistin, meropenem and rifampin in a combination therapy for multi-drug-resistant *Acinetobacter baumannii* multifocal infection. A case report. *Minerva. Anestesiol.* 73, 181–185.

Biglari, S., Hanafiah, A., Mohd Puzi, S., Ramli, R., Rahman, M., and Lopes, B. S. (2016). Antimicrobial resistance mechanisms and genetic diversity of multidrug-resistant *Acinetobacter baumannii* isolated from a teaching hospital in Malaysia. *Microb. Drug Resist.* doi: 10.1089/mdr.2016.0130. [Epub ahead of print].

Blanchard, C., Barnett, P., Perlmutter, J., and Dunman, P. M. (2014). Identification of *Acinetobacter baumannii* serum-associated antibiotic efflux pump inhibitors. *Antimicrob. Agents Chemother.* 58, 6360–6370. doi: 10.1128/AAC.03535-14

Bogaerts, P., Naas, T., El Garch, F., Cuzon, G., Deplano, A., Delaire, T., et al. (2010). GES extended-spectrum β-lactamases in *Acinetobacter baumannii* isolates in Belgium. *Antimicrob. Agents Chemother.* 54, 4872–4878. doi: 10.1128/AAC.00871-10

Boll, J. M., Tucker, A. T., Klein, D. R., Beltran, A. M., Brodbelt, J. S., Davies, B. W., et al. (2015). Reinforcing lipid A acylation on the cell surface of *Acinetobacter baumannii* promotes cationic antimicrobial peptide resistance and desiccation survival. *MBio* 6, e00478–e00415. doi: 10.1128/mBio.00478-15

Bonnin, R. A., Poirel, L., Licker, M., and Nordmann, P. (2011a). Genetic diversity of carbapenem-hydrolysing β-lactamases in *Acinetobacter baumannii* from Romanian hospitals. *Clin. Microbiol. Infect.* 17, 1524–1528. doi: 10.1111/j.1469-0691.2011.03622.x

Bonnin, R. A., Poirel, L., Naas, T., Pirs, M., Seme, K., Schrenzel, J., et al. (2012). Dissemination of New Delhi metallo-β-lactamases-1-producing *Acinetobacter baumannii* in Europe. *Clin. Microbiol. Infect.* 18, E362–E365. doi: 10.1111/j.1469-0691.2012.03928.x

Bonnin, R. A., Potron, A., Poirel, L., Lecuyer, H., Neri, R., and Nordmann, P. (2011b). PER-7, an extended-spectrum β-lactamases with increased activity toward broad-spectrum cephalosporins in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 55, 2424–2427. doi: 10.1128/AAC.01795-10

Borgeaud, S., Metzger, L. C., Scrignari, T., and Blokesch, M. (2015). The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science.* 347, 63–67. doi: 10.1126/science.1260064

Bou, G., Cervero, G., Dominguez, M. A., Quereda, C., and Martinez-Beltran, J. (2000a). Characterization of a nosocomial outbreak caused by a multiresistant *Acinetobacter baumannii* strain with a carbapenem-hydrolyzing enzyme: high-level carbapenem resistance in *A. baumannii* is not due solely to the presence of β-lactamases. *J. Clin. Microbiol.* 38, 3299–3305.

Bou, G., and Martinez-Beltran, J. (2000). Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC β-lactamases in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 44, 428–432. doi: 10.1128/AAC.44.2.428-432.2000

Bou, G., Oliver, A., and Martinez-Beltran, J. (2000b). OXA-24, a novel class D β-lactamases with carbapenemase activity in an *Acinetobacter baumannii* clinical strain. *Antimicrob. Agents Chemother.* 44, 1556–1561. doi: 10.1128/AAC.44.6.1556-1561.2000

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., et al. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* 48, 1–12. doi: 10.1086/595011

Bowler, S. L., Spychala, C. N., McElheny, C. L., Mettus, R. T., and Doi, Y. (2016). *In vitro* activity of fusidic acid-containing combinations against carbapenem-resistant *Acinetobacter baumannii* clinical strains. *Antimicrob. Agents Chemother.* 60, 5101. doi: 10.1128/AAC.01124-16

Briers, Y., Walmagh, M., Van Puyenbroeck, V., Cornelissen, A., Cenens, W., Aertsen, A., et al. (2014). Engineered endolysin-based "Artilysins" to combat multidrug-resistant gram-negative pathogens. *MBio* 5, e01379–14. doi: 10.1128/mBio.01379-14

Brigante, G., Migliavacca, R., Bramati, S., Motta, E., Nucleo, E., Manenti, M., et al. (2012). Emergence and spread of a multidrug-resistant *Acinetobacter baumannii* clone producing both the carbapenemase OXA-23 and the 16S rRNA methylase ArmA. *J. Med. Microbiol.* 61, 653–661. doi: 10.1099/jmm.0.040980-0

Brown, S., Young, H. K., and Amyes, S. G. (2005). Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. *Clin. Microbiol. Infect.* 11, 15–23. doi: 10.1111/j.1469-0691.2004.01016.x

Cai, Y., Chai, D., Wang, R., Liang, B., and Bai, N. (2012). Colistin resistance of *Acinetobacter baumannii*: clinical reports, mechanisms and antimicrobial strategies. *J. Antimicrob. Chemother.* 67, 1607–1615. doi: 10.1093/jac/dks084

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S., and Snyder, M. (2010). Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing. *PLoS Pathog.* 6:e1000834. doi: 10.1371/journal.ppat.1000834

Candel, F. J., Calvo, N., Head, J., Sanchez, A., Matesanz, M., Culebras, E., et al. (2010). A combination of tigecycline, colistin, and meropenem against multidrug-resistant *Acinetobacter baumannii* bacteremia in a renal transplant recipient: pharmacodynamic and microbiological aspects. *Rev. Esp. Quimioter.* 23, 103–108.

Cannon, J. P., Lee, T. A., Clark, N. M., Setlak, P., and Grim, S. A. (2014). The risk of seizures among the carbapenems: a meta-analysis. *J. Antimicrob. Chemother.* 69, 2043–2055. doi: 10.1093/jac/dku111

Carruthers, M. D., Nicholson, P. A., Tracy, E. N., and Munson, R. S. Jr. (2013). *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PLoS ONE* 8:e59388. doi: 10.1371/journal.pone.0059388

Castanheira, M., Mendes, R. E., and Jones, R. N. (2014). Update on *Acinetobacter* species: mechanisms of antimicrobial resistance and contemporary *in vitro* activity of minocycline and other treatment options. *Clin. Infect. Dis.* 59 (Suppl. 6), S367–S373. doi: 10.1093/cid/ciu706

Catel-Ferreira, M., Coadou, G., Molle, V., Mugnier, P., Nordmann, P., Siroy, A., et al. (2011). Structure-function relationships of CarO, the carbapenem resistance-associated outer membrane protein of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 66, 2053–2056. doi: 10.1093/jac/dkr267

Centers for Disease and Prevention (2004). *Acinetobacter baumannii* infections among patients at military medical facilities treating injured U.S. service members, 2002–2004. *MMWR Morb. Mortal. Wkly. Rep.* 53, 1063–1066.

Cerqueira, G. M., Kostoulias, X., Khoo, C., Aibinu, I., Qu, Y., Traven, A., et al. (2014). A global virulence regulator in *Acinetobacter baumannii* and its control of the phenylacetic acid catabolic pathway. *J. Infect. Dis.* 210, 46–55. doi: 10.1093/infdis/jiu024

Chagas, T. P., Carvalho, K. R., de Oliveira Santos, I. C., Carvalho-Assef, A. P., and Asensi, M. D. (2014). Characterization of carbapenem-resistant *Acinetobacter baumannii* in Brazil (2008-2011): countrywide spread of OXA-23-producing clones (CC15 and CC79). *Diagn. Microbiol. Infect. Dis.* 79, 468–472. doi: 10.1016/j.diagmicrobio.2014.03.006

Chang, H. C., Wei, Y. F., Dijkshoorn, L., Vaneechoutte, M., Tang, C. T., and Chang, T. C. (2005). Species-level identification of isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex by sequence analysis of the 16S-23S rRNA gene spacer region. *J. Clin. Microbiol.* 43, 1632–1639. doi: 10.1128/JCM.43.4.1632-1639.2005

Chang, K. C., Lin, M. F., Lin, N. T., Wu, W. J., Kuo, H. Y., Lin, T. Y., et al. (2012). Clonal spread of multidrug-resistant *Acinetobacter baumannii* in eastern Taiwan. *J. Microbiol. Immunol. Infect.* 45, 37–42. doi: 10.1016/j.jmii.2011.09.019

Cheah, S. E., Li, J., Tsuji, B. T., Forrest, A., Bulitta, J. B., and Nation, R. L. (2016). Colistin and polymyxin B dosage regimens against *Acinetobacter baumannii*: differences in activity and the emergence of resistance. *Antimicrob. Agents Chemother.* 60, 3921–3933. doi: 10.1128/AAC.02927-15

Chen, C. H., Young, T. G., and Huang, C. C. (2006). Predictive biomarkers for drug-resistant *Acinetobacter baumannii* isolates with $bla_{\text{TEM-1}}$, AmpC-type *bla* and integrase 1 genotypes. *J. Microbiol. Immunol. Infect.* 39, 372–379.

Chen, Q., Li, X., Zhou, H., Jiang, Y., Chen, Y., Hua, X., et al. (2014). Decreased susceptibility to tigecycline in *Acinetobacter baumannii* mediated by a mutation in *trm* encoding SAM-dependent methyltransferase. *J. Antimicrob. Chemother.* 69, 72–76. doi: 10.1093/jac/dkt319

Chen, T. L., Lee, Y. T., Kuo, S. C., Hsueh, P. R., Chang, F. Y., Siu, L. K., et al. (2010). Emergence and distribution of plasmids bearing the $bla_{\text{OXA-51}}$-like gene with an upstream IS*Aba1* in carbapenem-resistant *Acinetobacter baumannii* isolates in Taiwan. *Antimicrob. Agents Chemother.* 54, 4575–4581. doi: 10.1128/AAC.00764-10

Chen, T. L., Wu, R. C., Shaio, M. F., Fung, C. P., and Cho, W. L. (2008). Acquisition of a plasmid-borne $bla_{\text{OXA-58}}$ gene with an upstream IS*1008* insertion conferring a high level of carbapenem resistance to *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 52, 2573–2580. doi: 10.1128/AAC.00393-08

Chen, Y., Zhou, Z., Jiang, Y., and Yu, Y. (2011). Emergence of NDM-1-producing *Acinetobacter baumannii* in China. *J. Antimicrob. Chemother.* 66, 1255–1259. doi: 10.1093/jac/dkr082

Chihi, H., Bonnin, R. A., Bourouis, A., Mahrouki, S., Besbes, S., Moussa, M. B., et al. (2016). GES-11-producing *Acinetobacter baumannii* clinical

isolates from Tunisian hospitals: long-term dissemination of GES-type carbapenemases in North Africa. *J. Glob. Antimicrob. Resist.* 5, 47–50. doi: 10.1016/j.jgar.2016.03.005

Chin, C. Y., Gregg, K. A., Napier, B. A., Ernst, R. K., and Weiss, D. S. (2015). A PmrB-regulated deacetylase required for lipid A modification and polymyxin resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 59, 7911–7914. doi: 10.1128/AAC.00515-15

Chiu, C. H., Liu, Y. H., Wang, Y. C., Lee, Y. T., Kuo, S. C., Chen, T. L., et al. (2016). *In vitro* activity of SecA inhibitors in combination with carbapenems against carbapenem-hydrolysing class D β-lactamase-producing *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 71, 3441–3448. doi: 10.1093/jac/dkw331

Cho, Y. J., Moon, D. C., Jin, J. S., Choi, C. H., Lee, Y. C., and Lee, J. C. (2009). Genetic basis of resistance to aminoglycosides in *Acinetobacter* spp. and spread of *armA* in *Acinetobacter baumannii* sequence group 1 in Korean hospitals. *Diagn. Microbiol. Infect. Dis.* 64, 185–190. doi: 10.1016/j.diagmicrobio.2009.02.010

Choi, C. H., Hyun, S. H., Lee, J. Y., Lee, J. S., Lee, Y. S., Kim, S. A., et al. (2008a). *Acinetobacter baumannii* outer membrane protein A targets the nucleus and induces cytotoxicity. *Cell. Microbiol.* 10, 309–319. doi: 10.1111/j.1462-5822.2007.01041.x

Choi, C. H., Lee, E. Y., Lee, Y. C., Park, T. I., Kim, H. J., Hyun, S. H., et al. (2005). Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cell Microbiol.* 7, 1127–1138. doi: 10.1111/j.1462-5822.2005.00538.x

Choi, C. H., Lee, J. S., Lee, Y. C., Park, T. I., and Lee, J. C. (2008b). *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. *BMC Microbiol.* 8:216. doi: 10.1186/1471-2180-8-216

Chu, Y. W., Afzal-Shah, M., Houang, E. T., Palepou, M. I., Lyon, D. J., Woodford, N., et al. (2001). IMP-4, a novel metallo-β-lactamase from nosocomial *Acinetobacter* spp. collected in Hong Kong between 1994 and 1998. *Antimicrob. Agents Chemother.* 45, 710–714. doi: 10.1128/AAC.45.3.710-714.2001

Chung, J. H., Bhat, A., Kim, C. J., Yong, D., and Ryu, C. M. (2016). Combination therapy with polymyxin B and netropsin against clinical isolates of multidrug-resistant *Acinetobacter baumannii*. *Sci. Rep.* 6:28168. doi: 10.1038/srep28168

Cirioni, O., Simonetti, O., Pierpaoli, E., Barucca, A., Ghiselli, R., Orlando, F., et al. (2016). Colistin enhances therapeutic efficacy of daptomycin or teicoplanin in a murine model of multiresistant *Acinetobacter baumannii* sepsis. *Diagn. Microbiol. Infect. Dis.* 86, 392–398. doi: 10.1016/j.diagmicrobio.2016.09.010

Cirz, R. T., and Romesberg, F. E. (2007). Controlling mutation: intervening in evolution as a therapeutic strategy. *Crit. Rev. Biochem. Mol. Biol.* 42, 341–354. doi: 10.1080/10409230701597741

Cisneros, J. M., and Rodríguez-Baño, J. (2002). Nosocomial bacteremia due to *Acinetobacter baumannii*: epidemiology, clinical features and treatment. *Clin. Microbiol. Infect.* 8, 687–693. doi: 10.1046/j.1469-0691.2002.00487.x

Clemmer, K. M., Bonomo, R. A., and Rather, P. N. (2011). Genetic analysis of surface motility in *Acinetobacter baumannii*. *Microbiology* 157, 2534–2544. doi: 10.1099/mic.0.049791-0

Corbin, B. D., Seeley, E. H., Raab, A., Feldmann, J., Miller, M. R., Torres, V. J., et al. (2008). Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science* 319, 962–965. doi: 10.1126/science.1152449

Cordoba, J., Coronado-Alvarez, N. M., Parra, D., and Parra-Ruiz, J. (2015). *In vitro* activities of novel antimicrobial combinations against extensively drug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 59, 7316–7319. doi: 10.1128/AAC.00493-15

Corvec, S., Caroff, N., Espaze, E., Giraudeau, C., Drugeon, H., and Reynaud, A. (2003). AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *J. Antimicrob. Chemother.* 52, 629–635. doi: 10.1093/jac/dkg407

Corvec, S., Poirel, L., Naas, T., Drugeon, H., and Nordmann, P. (2007). Genetics and expression of the carbapenem-hydrolyzing oxacillinase gene bla_{OXA-23} in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 51, 1530–1533. doi: 10.1128/AAC.01132-06

Coyne, S., Courvalin, P., and Perichon, B. (2011). Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrob. Agents Chemother.* 55, 947–953. doi: 10.1128/AAC.01388-10

Coyne, S., Rosenfeld, N., Lambert, T., Courvalin, P., and Perichon, B. (2010). Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 54, 4389–4393. doi: 10.1128/AAC.00155-10

Croxatto, A., Prod'hom, G., and Greub, G. (2012). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol. Rev.* 36, 380–407. doi: 10.1111/j.1574-6976.2011.00298.x

Curcio, D., and Fernandez, F. (2008). Tigecycline for *Acinetobacter baumannii* infection: other considerations. *Clin. Infect. Dis.* 46, 1797–1798; author reply 1798–1799. doi: 10.1086/588051

Damier-Piolle, L., Magnet, S., Bremont, S., Lambert, T., and Courvalin, P. (2008). AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 52, 557–562. doi: 10.1128/AAC.00732-07

Davison, M., Treangen, T. J., Koren, S., Pop, M., and Bhaya, D. (2016). Diversity in a polymicrobial community revealed by analysis of viromes, endolysins and CRISPR spacers. *PLoS ONE* 11:e0160574. doi: 10.1371/journal.pone.0160574

de Breij, A., Dijkshoorn, L., Lagendijk, E., van der Meer, J., Koster, A., Bloemberg, G., et al. (2010). Do biofilm formation and interactions with human cells explain the clinical success of *Acinetobacter baumannii*? *PLoS ONE* 5:e10732. doi: 10.1371/journal.pone.0010732

Defraine, V., Schuermans, J., Grymonprez, B., Govers, S. K., Aertsen, A., Fauvart, M., et al. (2016). Efficacy of artilysin Art-175 against resistant and persistent *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 3480–3488. doi: 10.1128/AAC.00285-16

del Mar Tomas, M., Beceiro, A., Perez, A., Velasco, D., Moure, R., Villanueva, R., et al. (2005). Cloning and functional analysis of the gene encoding the 33- to 36-kilodalton outer membrane protein associated with carbapenem resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 49, 5172–5175. doi: 10.1128/AAC.49.12.5172-5175.2005

Deng, M., Zhu, M. H., Li, J. J., Bi, S., Sheng, Z. K., Hu, F. S., et al. (2014). Molecular epidemiology and mechanisms of tigecycline resistance in clinical isolates of *Acinetobacter baumannii* from a Chinese university hospital. *Antimicrob. Agents Chemother.* 58, 297–303. doi: 10.1128/AAC.01727-13

de Sa Cavalcanti, F. L., Mendes-Marques, C. L., Vasconcelos, C. R., de Lima Campos, T., Rezende, A. M., Xavier, D. E., et al. (2016). High frequency of OXA-253-producing *Acinetobacter baumannii* in different hospitals in Recife, Brazil: a new threat? *Antimicrob. Agents Chemother.* 61:e01309-16. doi: 10.1128/AAC.01309-16

Dhabaan, G. N., AbuBakar, S., Cerqueira, G. M., Al-Haroni, M., Pang, S. P., and Hassan, H. (2015). Imipenem treatment induces expression of important genes and phenotypes in a resistant *Acinetobacter baumannii* isolate. *Antimicrob. Agents Chemother.* 60, 1370–1376. doi: 10.1128/AAC.01696-15

Dijkshoorn, L., Aucken, H., Gerner-Smidt, P., Janssen, P., Kaufmann, M. E., Garaizar, J., et al. (1996). Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. *J. Clin. Microbiol.* 34, 1519–1525.

Dizbay, M., Tozlu, D. K., Cirak, M. Y., Isik, Y., Ozdemir, K., and Arman, D. (2010). *In vitro* synergistic activity of tigecycline and colistin against XDR-*Acinetobacter baumannii*. *J. Antibiot. (Tokyo)* 63, 51–53. doi: 10.1038/ja.2009.117

Doi, Y., Murray, G. L., and Peleg, A. Y. (2015). *Acinetobacter baumannii*: evolution of antimicrobial resistance-treatment options. *Semin. Respir. Crit. Care Med.* 36, 85–98. doi: 10.1055/s-0034-1398388

Doi, Y., Wachino, J., Yamane, K., Shibata, N., Yagi, T., Shibayama, K., et al. (2004). Spread of novel aminoglycoside resistance gene *aac(6′)*-Iad among *Acinetobacter* clinical isolates in Japan. *Antimicrob. Agents Chemother.* 48, 2075–2080. doi: 10.1128/AAC.48.6.2075-2080.2004

Donnarumma, F., Sergi, S., Indorato, C., Mastromei, G., Monnanni, R., Nicoletti, P., et al. (2010). Molecular characterization of acinetobacter isolates collected in intensive care units of six hospitals in Florence, Italy, during a 3-year surveillance program: a population structure analysis. *J. Clin. Microbiol.* 48, 1297–1304. doi: 10.1128/JCM.01916-09

Dortet, L., Bonnin, R. A., Bernabeu, S., Escaut, L., Vittecoq, D., Girlich, D., et al. (2016). First occurrence of OXA-72-producing *Acinetobacter baumannii* in Serbia. *Antimicrob. Agents Chemother.* 60, 5724–5730. doi: 10.1128/AAC.01016-16

Dubois, D., Prasadarao, N. V., Mittal, R., Bret, L., Roujou-Gris, M., and Bonnet, R. (2009). CTX-M β-lactamase production and virulence of *Escherichia coli* K1. *Emerg. Infect. Dis.* 15, 1988–1990. doi: 10.3201/eid1512.090928

Dubrovskaya, Y., Prasad, N., Lee, Y., Esaian, D., Figueroa, D. A., and Tam, V. H. (2015). Risk factors for nephrotoxicity onset associated with polymyxin B therapy. *J. Antimicrob. Chemother.* 70, 1903–1907. doi: 10.1093/jac/dkv014

Dupont, M., Pages, J. M., Lafitte, D., Siroy, A., and Bollet, C. (2005). Identification of an OprD homologue in *Acinetobacter baumannii*. *J. Proteome. Res.* 4, 2386–2390. doi: 10.1021/pr050143q

Elhosseiny, N. M., Amin, M. A., Yassin, A. S., and Attia, A. S. (2015). *Acinetobacter baumannii* universal stress protein A plays a pivotal role in stress response and is essential for pneumonia and sepsis pathogenesis. *Int. J. Med. Microbiol.* 305, 114–123. doi: 10.1016/j.ijmm.2014.11.008

Elhosseiny, N. M., El-Tayeb, O. M., Yassin, A. S., Lory, S., and Attia, A. S. (2016). The secretome of *Acinetobacter baumannii* ATCC 17978 type II secretion system reveals a novel plasmid encoded phospholipase that could be implicated in lung colonization. *Int. J. Med. Microbiol.* 306, 633–641. doi: 10.1016/j.ijmm.2016.09.006

Ellis, T. N., and Kuehn, M. J. (2010). Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* 74, 81–94. doi: 10.1128/MMBR.00031-09

Endimiani, A., Luzzaro, F., Migliavacca, R., Mantengoli, E., Hujer, A. M., Hujer, K. M., et al. (2007). Spread in an Italian hospital of a clonal *Acinetobacter baumannii* strain producing the TEM-92 extended-spectrum β-lactamase. *Antimicrob. Agents Chemother.* 51, 2211–2214. doi: 10.1128/AAC.01139-06

Erridge, C., Moncayo-Nieto, O. L., Morgan, R., Young, M., and Poxton, I. R. (2007). *Acinetobacter baumannii* lipopolysaccharides are potent stimulators of human monocyte activation via Toll-like receptor 4 signalling. *J. Med. Microbiol.* 56, 165–171. doi: 10.1099/jmm.0.46823-0

Espinal, P., Fugazza, G., Lopez, Y., Kasma, M., Lerman, Y., Malhotra-Kumar, S., et al. (2011). Dissemination of an NDM-2-producing *Acinetobacter baumannii* clone in an Israeli rehabilitation center. *Antimicrob. Agents Chemother.* 55, 5396–5398. doi: 10.1128/AAC.00679-11

Evans, B. A., Brown, S., Hamouda, A., Findlay, J., and Amyes, S. G. (2007). Eleven novel OXA-51-like enzymes from clinical isolates of *Acinetobacter baumannii*. *Clin. Microbiol. Infect.* 13, 1137–1138. doi: 10.1111/j.1469-0691.2007.01828.x

Fajardo Bonin, R., Chapeaurouge, A., Perales, J., da Silva, J. G. Jr., do Nascimento, H. J., D'Alincourt Carvalho Assef, A. P., et al. (2014). Identification of immunogenic proteins of the bacterium *Acinetobacter baumannii* using a proteomic approach. *Proteomics Clin. Appl.* 8, 916–923. doi: 10.1002/prca.201300133

Fan, B., Guan, J., Wang, X., and Cong, Y. (2016). Activity of colistin in combination with meropenem, tigecycline, fosfomycin, fusidic acid, rifampin or sulbactam against extensively drug-resistant *Acinetobacter baumannii* in a murine thigh-infection model. *PLoS ONE* 11:e0157757. doi: 10.1371/journal.pone.0157757

Fang, F., Wang, S., Dang, Y. X., Wang, X., and Yu, G. Q. (2016). Molecular characterization of carbapenemase genes in *Acinetobacter baumannii* in China. *Genet. Mol. Res.* 15:gmr7432. doi: 10.4238/gmr.15017432

Fernandez-Cuenca, F., Smani, Y., Gomez-Sanchez, M. C., Docobo-Perez, F., Caballero-Moyano, F. J., Dominguez-Herrera, J., et al. (2011). Attenuated virulence of a slow-growing pandrug-resistant *Acinetobacter baumannii* is associated with decreased expression of genes encoding the porins CarO and OprD-like. *Int. J. Antimicrob. Agents* 38, 548–549. doi: 10.1016/j.ijantimicag.2011.08.002

Fernando, D., and Kumar, A. (2012). Growth phase-dependent expression of RND efflux pump- and outer membrane porin-encoding genes in *Acinetobacter baumannii* ATCC 19606. *J. Antimicrob. Chemother.* 67, 569–572. doi: 10.1093/jac/dkr519

Fiester, S. E., Arivett, B. A., Schmidt, R. E., Beckett, A. C., Ticak, T., Carrier, M. V., et al. (2016). Iron-regulated phospholipase C activity contributes to the cytolytic activity and virulence of *Acinetobacter baumannii*. *PLoS ONE* 11:e0167068. doi: 10.1371/journal.pone.0167068

Flores-Diaz, M., Monturiol-Gross, L., Naylor, C., Alape-Giron, A., and Flieger, A. (2016). Bacterial sphingomyelinases and phospholipases as virulence factors. *Microbiol. Mol. Biol. Rev.* 80, 597–628. doi: 10.1128/MMBR.00082-15

Fonseca, E. L., Scheidegger, E., Freitas, F. S., Cipriano, R., and Vicente, A. C. (2013). Carbapenem-resistant *Acinetobacter baumannii* from Brazil: role of *carO* alleles expression and bla_{OXA-23} gene. *BMC Microbiol.* 13:245. doi: 10.1186/1471-2180-13-245

Fournier, P. E., and Richet, H. (2006). The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clin. Infect. Dis.* 42, 692–699. doi: 10.1086/500202

Fournier, P. E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., et al. (2006). Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet.* 2:e7. doi: 10.1371/journal.pgen.0020007

Fulnecky, E. J., Wright, D., Scheld, W. M., Kanawati, L., and Shoham, S. (2005). Amikacin and colistin for treatment of *Acinetobacter baumannii* meningitis. *J. Infect.* 51, e249–e251. doi: 10.1016/j.jinf.2005.04.003

Gaddy, J. A., Arivett, B. A., McConnell, M. J., Lopez-Rojas, R., Pachon, J., and Actis, L. A. (2012). Role of acinetobactin-mediated iron acquisition functions in the interaction of *Acinetobacter baumannii* strain ATCC 19606T with human lung epithelial cells, *Galleria mellonella* caterpillars, and mice. *Infect. Immun.* 80, 1015–1024. doi: 10.1128/IAI.06279-11

Gaddy, J. A., Tomaras, A. P., and Actis, L. A. (2009). The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect. Immun.* 77, 3150–3160. doi: 10.1128/IAI.00096-09

Gales, A. C., Tognim, M. C., Reis, A. O., Jones, R. N., and Sader, H. S. (2003). Emergence of an IMP-like metallo-enzyme in an *Acinetobacter baumannii* clinical strain from a Brazilian teaching hospital. *Diagn. Microbiol. Infect. Dis.* 45, 77–79. doi: 10.1016/S0732-8893(02)00500-X

Gallego, L., and Towner, K. J. (2001). Carriage of class 1 integrons and antibiotic resistance in clinical isolates of *Acinetobacter baumannii* from northern Spain. *J. Med. Microbiol.* 50, 71–77. doi: 10.1099/0022-1317-50-1-71

Garcia-Quintanilla, M., Carretero-Ledesma, M., Moreno-Martinez, P., Martin-Pena, R., Pachon, J., and McConnell, M. J. (2015). Lipopolysaccharide loss produces partial colistin dependence and collateral sensitivity to azithromycin, rifampicin and vancomycin in *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents* 46, 696–702. doi: 10.1016/j.ijantimicag.2015.07.017

Garcia-Salguero, C., Rodriguez-Avial, I., Picazo, J. J., and Culebras, E. (2015). Can plazomicin alone or in combination be a therapeutic pption against carbapenem-resistant *Acinetobacter baumannii*? *Antimicrob. Agents Chemother.* 59, 5959–5966. doi: 10.1128/AAC.00873-15

Gebhardt, M. J., Gallagher, L. A., Jacobson, R. K., Usacheva, E. A., Peterson, L. R., Zurawski, D. V., et al. (2015). Joint transcriptional control of virulence and resistance to antibiotic and environmental stress in *Acinetobacter baumannii*. *MBio* 6, e01660–15. doi: 10.1128/mBio.01660-15

Gehrlein, M., Leying, H., Cullmann, W., Wendt, S., and Opferkuch, W. (1991). Imipenem resistance in *Acinetobacter baumanii* is due to altered penicillin-binding proteins. *Chemotherapy* 37, 405–412.

Geisinger, E., and Isberg, R. R. (2015). Antibiotic modulation of capsular exopolysaccharide and virulence in *Acinetobacter baumannii*. *PLoS Pathog.* 11:e1004691. doi: 10.1371/journal.ppat.1004691

Giannouli, M., Tomasone, F., Agodi, A., Vahaboglu, H., Daoud, Z., Triassi, M., et al. (2009). Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* strains in intensive care units of multiple Mediterranean hospitals. *J. Antimicrob. Chemother.* 63, 828–830. doi: 10.1093/jac/dkp032

Gogou, V., Pournaras, S., Giannouli, M., Voulgari, E., Piperaki, E. T., Zarrilli, R., et al. (2011). Evolution of multidrug-resistant *Acinetobacter baumannii* clonal lineages: a 10 year study in Greece (2000–09). *J. Antimicrob. Chemother.* 66, 2767–2772. doi: 10.1093/jac/dkr390

Goic-Barisic, I., Towner, K. J., Kovacic, A., Sisko-Kraljevic, K., Tonkic, M., Novak, A., et al. (2011). Outbreak in Croatia caused by a new carbapenem-resistant clone of *Acinetobacter baumannii* producing OXA-72 carbapenemase. *J. Hosp. Infect.* 77, 368–369. doi: 10.1016/j.jhin.2010.12.003

Gonzalez-Villoria, A. M., Tamayo-Legorreta, E., Garza-Ramos, U., Barrios, H., Sanchez-Perez, A., Rodriguez-Medina, N., et al. (2016). A multicenter study in Mexico finds *Acinetobacter baumannii* clinical isolates belonging to clonal complexes 636B (113B) and 92B harboring OXA-72, OXA-239, and OXA-469. *Antimicrob. Agents Chemother.* 60, 2587–2588. doi: 10.1128/AAC.02042-15

Gordon, N. C., and Wareham, D. W. (2009). A review of clinical and microbiological outcomes following treatment of infections involving multidrug-resistant *Acinetobacter baumannii* with tigecycline. *J. Antimicrob. Chemother.* 63, 775–780. doi: 10.1093/jac/dkn555

Gordon, N. C., and Wareham, D. W. (2010). Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int. J. Antimicrob. Agents* 35, 219–226. doi: 10.1016/j.ijantimicag.2009.10.024

Hagihara, M., Housman, S. T., Nicolau, D. P., and Kuti, J. L. (2014). *In vitro* pharmacodynamics of polymyxin B and tigecycline alone and in combination against carbapenem-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 58, 874–879. doi: 10.1128/AAC.01624-13

Hammerstrom, T. G., Beabout, K., Clements, T. P., Saxer, G., and Shamoo, Y. (2015). *Acinetobacter baumannii* repeatedly evolves a hypermutator phenotype

in response to tigecycline that effectively surveys evolutionary trajectories to resistance. *PLoS ONE* 10:e0140489. doi: 10.1371/journal.pone.0140489

Hamouda, A., Evans, B. A., Towner, K. J., and Amyes, S. G. (2010). Characterization of epidemiologically unrelated *Acinetobacter baumannii* isolates from four continents by use of multilocus sequence typing, pulsed-field gel electrophoresis, and sequence-based typing of $bla_{\text{OXA-51-like}}$ genes. *J. Clin. Microbiol.* 48, 2476–2483. doi: 10.1128/JCM.02431-09

Harding, C. M., Kinsella, R. L., Palmer, L. D., Skaar, E. P., and Feldman, M. F. (2016). Medically relevant *Acinetobacter* species require a type II secretion system and specific membrane-associated chaperones for the export of multiple substrates and full virulence. *PLoS Pathog.* 12:e1005391. doi: 10.1371/journal.ppat.1005391

Hasani, A., Sheikhalizadeh, V., Ahangarzadeh Rezaee, M., Rahmati-Yamchi, M., Hasani, A., Ghotaslou, R., et al. (2016). Frequency of aminoglycoside-modifying enzymes and ArmA among different sequence groups of *Acinetobacter baumannii* in Iran. *Microb. Drug Resist.* 22, 347–353. doi: 10.1089/mdr.2015.0254

Hassan, A., Naz, A., Obaid, A., Paracha, R. Z., Naz, K., Awan, F. M., et al. (2016). Pangenome and immuno-proteomics analysis of *Acinetobacter baumannii* strains revealed the core peptide vaccine targets. *BMC Genomics.* 17:732. doi: 10.1186/s12864-016-2951-4

Hawley, J. S., Murray, C. K., and Jorgensen, J. H. (2007). Development of colistin-dependent *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex. *Antimicrob. Agents Chemother.* 51, 4529–4530. doi: 10.1128/AAC.01115-07

He, S., He, H., Chen, Y., Chen, Y., Wang, W., and Yu, D. (2015). *In vitro* and *in vivo* analysis of antimicrobial agents alone and in combination against multi-drug resistant *Acinetobacter baumannii*. *Front. Microbiol.* 6, 507. doi: 10.3389/fmicb.2015.00507

He, X., Lu, F., Yuan, F., Jiang, D., Zhao, P., Zhu, J., et al. (2015). Biofilm formation caused by clinical *Acinetobacter baumannii* isolates is associated with overexpression of the AdeFGH efflux pump. *Antimicrob. Agents Chemother.* 59, 4817–4825. doi: 10.1128/AAC.00877-15

Heritier, C., Poirel, L., Aubert, D., and Nordmann, P. (2003). Genetic and functional analysis of the chromosome-encoded carbapenem-hydrolyzing oxacillinase OXA-40 of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 47, 268–273. doi: 10.1128/AAC.47.1.268-273.2003

Heritier, C., Poirel, L., Fournier, P. E., Claverie, J. M., Raoult, D., and Nordmann, P. (2005a). Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 49, 4174–4179. doi: 10.1128/AAC.49.10.4174-4179.2005

Heritier, C., Poirel, L., Lambert, T., and Nordmann, P. (2005b). Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 49, 3198–3202. doi: 10.1128/AAC.49.8.3198-3202.2005

Heritier, C., Poirel, L., and Nordmann, P. (2006). Cephalosporinase over-expression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii*. *Clin. Microbiol. Infect.* 12, 123–130. doi: 10.1111/j.1469-0691.2005.01320.x

Higgins, P. G., Janssen, K., Fresen, M. M., Wisplinghoff, H., and Seifert, H. (2012). Molecular epidemiology of *Acinetobacter baumannii* bloodstream isolates obtained in the United States from 1995 to 2004 using rep-PCR and multilocus sequence typing. *J. Clin. Microbiol.* 50, 3493–3500. doi: 10.1128/JCM.01759-12

Higgins, P. G., Perez-Llarena, F. J., Zander, E., Fernandez, A., Bou, G., and Seifert, H. (2013). OXA-235, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 57, 2121–2126. doi: 10.1128/AAC.02413-12

Higgins, P. G., Poirel, L., Lehmann, M., Nordmann, P., and Seifert, H. (2009). OXA-143, a novel carbapenem-hydrolyzing class D β-lactamase in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53, 5035–5038. doi: 10.1128/AAC.00856-09

Higgins, P. G., Wisplinghoff, H., Stefanik, D., and Seifert, H. (2004). Selection of topoisomerase mutations and overexpression of *adeB* mRNA transcripts during an outbreak of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 54, 821–823. doi: 10.1093/jac/dkh427

Hiraki, Y., Yoshida, M., Masuda, Y., Inoue, D., Tsuji, Y., Kamimura, H., et al. (2013). Successful treatment of skin and soft tissue infection due to carbapenem-resistant *Acinetobacter baumannii* by ampicillin-sulbactam and meropenem combination therapy. *Int. J. Infect. Dis.* 17, e1234–e1236. doi: 10.1016/j.ijid.2013.05.002

Hogg, G. M., Barr, J. G., and Webb, C. H. (1998). *In-vitro* activity of the combination of colistin and rifampicin against multidrug-resistant strains of *Acinetobacter baumannii*. *J Antimicrob Chemother.* 41, 494-495.

Hong, S. B., Shin, K. S., Ha, J., and Han, K. (2013). Co-existence of $bla_{\text{OXA-23}}$ and *armA* in multidrug-resistant *Acinetobacter baumannii* isolated from a hospital in South Korea. *J. Med. Microbiol.* 62, 836–844. doi: 10.1099/jmm.0.055384-0

Hong, Y. K., Lee, J. Y., Wi, Y. M., and Ko, K. S. (2016). High rate of colistin dependence in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 71, 2346–2348. doi: 10.1093/jac/dkw121

Hood, M. I., Jacobs, A. C., Sayood, K., Dunman, P. M., and Skaar, E. P. (2010). *Acinetobacter baumannii* increases tolerance to antibiotics in response to monovalent cations. *Antimicrob. Agents Chemother.* 54, 1029–1041. doi: 10.1128/AAC.00963-09

Hood, M. I., Mortensen, B. L., Moore, J. L., Zhang, Y., Kehl-Fie, T. E., Sugitani, N., et al. (2012). Identification of an *Acinetobacter baumannii* zinc acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration. *PLoS Pathog.* 8:e1003068. doi: 10.1371/journal.ppat.1003068

Hornsey, M., Ellington, M. J., Doumith, M., Thomas, C. P., Gordon, N. C., Wareham, D. W., et al. (2010). AdeABC-mediated efflux and tigecycline MICs for epidemic clones of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 65, 1589–1593. doi: 10.1093/jac/dkq218

Hornsey, M., Loman, N., Wareham, D. W., Ellington, M. J., Pallen, M. J., Turton, J. F., et al. (2011). Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy. *J. Antimicrob. Chemother.* 66, 1499–1503. doi: 10.1093/jac/dkr168

Hornsey, M., and Wareham, D. W. (2011). *In vivo* efficacy of glycopeptide-colistin combination therapies in a *Galleria mellonella* model of *Acinetobacter baumannii* infection. *Antimicrob. Agents Chemother.* 55, 3534–3537. doi: 10.1128/AAC.00230-11

Hou, C., and Yang, F. (2015). Drug-resistant gene of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$and $bla_{\text{OXA-58}}$ in *Acinetobacter baumannii*. *Int. J. Clin. Exp. Med.* 8, 13859–13863.

Houang, E. T., Chu, Y. W., Chu, K. Y., Ng, K. C., Leung, C. M., and Cheng, A. F. (2003). Significance of genomic DNA group delineation in comparative studies of antimicrobial susceptibility of *Acinetobacter* spp. *Antimicrob. Agents Chemother.* 47, 1472–1475. doi: 10.1128/AAC.47.4.1472-1475.2003

Hu, D., Liu, B., Dijkshoorn, L., Wang, L., and Reeves, P. R. (2013). Diversity in the major polysaccharide antigen of *Acinetobacter baumannii* assessed by DNA sequencing, and development of a molecular serotyping scheme. *PLoS ONE* 8:e70329. doi: 10.1371/journal.pone.0070329

Hu, W. S., Yao, S. M., Fung, C. P., Hsieh, Y. P., Liu, C. P., and Lin, J. F. (2007). An OXA-66/OXA-51-like carbapenemase and possibly an efflux pump are associated with resistance to imipenem in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 51, 3844–3852. doi: 10.1128/AAC.01512-06

Huang, W., Yao, Y., Long, Q., Yang, X., Sun, W., Liu, C., et al. (2014). Immunization against multidrug-resistant *Acinetobacter baumannii* effectively protects mice in both pneumonia and sepsis models. *PLoS ONE* 9:e100727. doi: 10.1371/journal.pone.0100727

Huang, W., Yao, Y., Wang, S., Xia, Y., Yang, X., Long, Q., et al. (2016). Immunization with a 22-kDa outer membrane protein elicits protective immunity to multidrug-resistant *Acinetobacter baumannii*. *Sci. Rep.* 6:20724. doi: 10.1038/srep20724

Hujer, K. M., Hamza, N. S., Hujer, A. M., Perez, F., Helfand, M. S., Bethel, C. R., et al. (2005). Identification of a new allelic variant of the *Acinetobacter baumannii* cephalosporinase, ADC-7 β-lactamase: defining a unique family of class C enzymes. *Antimicrob. Agents Chemother.* 49, 2941–2948. doi: 10.1128/AAC.49.7.2941-2948.2005

Iwashkiw, J. A., Seper, A., Weber, B. S., Scott, N. E., Vinogradov, E., Stratilo, C., et al. (2012). Identification of a general O-linked protein glycosylation system in *Acinetobacter baumannii* and its role in virulence and biofilm formation. *PLoS Pathog.* 8:e1002758. doi: 10.1371/journal.ppat.1002758

Jacobs, A. C., Hood, I., Boyd, K. L., Olson, P. D., Morrison, J. M., Carson, S., et al. (2010). Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infect. Immun.* 78, 1952–1962. doi: 10.1128/IAI.00889-09

Jara, L. M., Perez-Varela, M., Corral, J., Arch, M., Cortes, P., Bou, G., et al. (2015). Novobiocin inhibits the antimicrobial resistance acquired through DNA damage-induced mutagenesis in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 637–639. doi: 10.1128/AAC.01810-15

Jaruratanasirikul, S., Wongpoowarak, W., Wattanavijitkul, T., Sukarnjanaset, W., Samaeng, M., Nawakitrangsan, M., et al. (2016). Population pharmacokinetics and pharmacodynamics modeling to optimize dosage regimens of sulbactam in critically ill patients with severe sepsis caused by *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 7236–7244. doi: 10.1128/AAC.01669-16

Jeon, J. H., Lee, J. H., Lee, J. J., Park, K. S., Karim, A. M., Lee, C. R., et al. (2015). Structural basis for carbapenem-hydrolyzing mechanisms of carbapenemases conferring antibiotic resistance. *Int. J. Mol. Sci.* 16, 9654–9692. doi: 10.3390/ijms16059654

Jeon, J., Ryu, C. M., Lee, J. Y., Park, J. H., Yong, D., and Lee, K. (2016). *In vivo* application of bacteriophage as a potential therapeutic agent to control OXA-66-like carbapenemase-producing *Acinetobacter baumannii* strains belonging to sequence type 357. *Appl. Environ. Microbiol.* 82, 4200–4208. doi: 10.1128/AEM.00526-16

Jeong, H. W., Cheong, H. J., Kim, W. J., Kim, M. J., Song, K. J., Song, J. W., et al. (2009). Loss of the 29-kilodalton outer membrane protein in the presence of OXA-51-like enzymes in *Acinetobacter baumannii* is associated with decreased imipenem susceptibility. *Microb. Drug Resist.* 15, 151–158. doi: 10.1089/mdr.2009.0828

Jeong, S. H., Bae, I. K., Kwon, S. B., Lee, K., Yong, D., Woo, G. J., et al. (2005). Investigation of a nosocomial outbreak of *Acinetobacter baumannii* producing PER-1 extended-spectrum β-lactamase in an intensive care unit. *J. Hosp. Infect.* 59, 242–248. doi: 10.1016/j.jhin.2004.09.025

Jin, J. S., Kwon, S. O., Moon, D. C., Gurung, M., Lee, J. H., Kim, S. I., et al. (2011). *Acinetobacter baumannii* secretes cytotoxic outer membrane protein A via outer membrane vesicles. *PLoS ONE* 6:e17027. doi: 10.1371/journal.pone.0017027

Johnson, T. L., Waack, U., Smith, S., Mobley, H., and Sandkvist, M. (2015). *Acinetobacter baumannii* is dependent on the type II secretion system and its substrate LipA for lipid utilization and *in vivo* fitness. *J. Bacteriol.* 198, 711–719. doi: 10.1128/JB.00622-15

Jones, C. L., Clancy, M., Honnold, C., Singh, S., Snesrud, E., Onmus-Leone, F., et al. (2015). Fatal outbreak of an emerging clone of extensively drug-resistant *Acinetobacter baumannii* with enhanced virulence. *Clin. Infect. Dis.* 61, 145–154. doi: 10.1093/cid/civ225

Jun, S. H., Lee, J. H., Kim, B. R., Kim, S. I., Park, T. I., Lee, J. C., et al. (2013). *Acinetobacter baumannii* outer membrane vesicles elicit a potent innate immune response via membrane proteins. *PLoS ONE* 8:e71751. doi: 10.1371/journal.pone.0071751

Juttukonda, L. J., Chazin, W. J., and Skaar, E. P. (2016). *Acinetobacter baumannii* coordinates urea metabolism with metal import to resist host-mediated metal limitation. *MBio* 7:e01475-16. doi: 10.1128/mBio.01475-16

Kalin, G., Alp, E., Akin, A., Coskun, R., and Doganay, M. (2014). Comparison of colistin and colistin/sulbactam for the treatment of multidrug resistant *Acinetobacter baumannii* ventilator-associated pneumonia. *Infection.* 42, 37–42. doi: 10.1007/s15010-013-0495-y

Kaplan, J. B. (2011). Antibiotic-induced biofilm formation. *Int. J. Artif. Organs.* 34, 737–751. doi: 10.5301/ijao.5000027

Karthikeyan, K., Thirunarayan, M. A., and Krishnan, P. (2010). Coexistence of $bla_{\text{OXA-23}}$ with $bla_{\text{NDM-1}}$ and *armA* in clinical isolates of *Acinetobacter baumannii* from India. *J. Antimicrob. Chemother.* 65, 2253–2254. doi: 10.1093/jac/dkq273

Kenyon, J. J., and Hall, R. M. (2013). Variation in the complex carbohydrate biosynthesis loci of *Acinetobacter baumannii* genomes. *PLoS ONE* 8:e62160. doi: 10.1371/journal.pone.0062160

Kim, C. K., Lee, Y., Lee, H., Woo, G. J., Song, W., Kim, M. N., et al. (2010). Prevalence and diversity of carbapenemases among imipenem-nonsusceptible *Acinetobacter* isolates in Korea: emergence of a novel OXA-182. *Diagn. Microbiol. Infect. Dis.* 68, 432–438. doi: 10.1016/j.diagmicrobio.2010.07.014

Kim, S. W., Choi, C. H., Moon, D. C., Jin, J. S., Lee, J. H., Shin, J. H., et al. (2009). Serum resistance of *Acinetobacter baumannii* through the binding of factor H to outer membrane proteins. *FEMS Microbiol. Lett.* 301, 224–231. doi: 10.1111/j.1574-6968.2009.01820.x

Kim, Y. J., Kim, S. I., Kim, Y. R., Hong, K. W., Wie, S. H., Park, Y. J., et al. (2012). Carbapenem-resistant *Acinetobacter baumannii*: diversity of resistant mechanisms and risk factors for infection. *Epidemiol. Infect.* 140, 137–145. doi: 10.1017/S0950268811000744

Knight, D., Dimitrova, D. D., Rudin, S. D., Bonomo, R. A., and Rather, P. N. (2016). Mutations decreasing intrinsic β-lactam resistance are linked to cell division in the nosocomial pathogen *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 3751–3758. doi: 10.1128/AAC.00361-16

Kobayashi, N., Nishino, K., and Yamaguchi, A. (2001). Novel macrolide-specific ABC-type efflux transporter in *Escherichia coli*. *J. Bacteriol.* 183, 5639–5644. doi: 10.1128/JB.183.19.5639-5644.2001

Koeleman, J. G., Stoof, J., Biesmans, D. J., Savelkoul, P. H., and Vandenbroucke-Grauls, C. M. (1998). Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii*. *J. Clin. Microbiol.* 36, 2522–2529.

Koeleman, J. G., van der Bijl, M. W., Stoof, J., Vandenbroucke-Grauls, C. M., and Savelkoul, P. H. (2001). Antibiotic resistance is a major risk factor for epidemic behavior of *Acinetobacter baumannii*. *Infect. Control Hosp Epidemiol.* 22, 284–288. doi: 10.1086/501901

Koenigs, A., Stahl, J., Averhoff, B., Gottig, S., Wichelhaus, T. A., Wallich, R., et al. (2016). CipA of *Acinetobacter baumannii* is a novel plasminogen binding and complement inhibitory protein. *J. Infect. Dis.* 213, 1388–1399. doi: 10.1093/infdis/jiv601

Koenigs, A., Zipfel, P. F., and Kraiczy, P. (2015). Translation elongation factor Tuf of *Acinetobacter baumannii* is a plasminogen-binding protein. *PLoS ONE* 10:e0134418. doi: 10.1371/journal.pone.0134418

Koh, T. H., Sng, L. H., Wang, G. C., Hsu, L. Y., and Zhao, Y. (2007). IMP-4 and OXA beta-lactamases in *Acinetobacter baumannii* from Singapore. *J. Antimicrob. Chemother.* 59, 627–632. doi: 10.1093/jac/dkl544

Koh, T. H., Tan, T. T., Khoo, C. T., Ng, S. Y., Tan, T. Y., Hsu, L. Y., et al. (2012). *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species in clinical specimens in Singapore. *Epidemiol. Infect.* 140, 535–538. doi: 10.1017/S0950268811001129

Kohlenberg, A., Brummer, S., Higgins, P. G., Sohr, D., Piening, B. C., de Grahl, C., et al. (2009). Outbreak of carbapenem-resistant *Acinetobacter baumannii* carrying the carbapenemase OXA-23 in a German university medical centre. *J. Med. Microbiol.* 58, 1499–1507. doi: 10.1099/jmm.0.012302-0

Konstantinidis, T., Kambas, K., Mitsios, A., Panopoulou, M., Tsironidou, V., Dellaporta, E., et al. (2016). Immunomodulatory role of clarithromycin in *Acinetobacter baumannii* infection via formation of neutrophil extracellular traps. *Antimicrob. Agents Chemother.* 60, 1040–1048. doi: 10.1128/AAC.02063-15

Koomanachai, P., Kim, A., and Nicolau, D. P. (2009). Pharmacodynamic evaluation of tigecycline against *Acinetobacter baumannii* in a murine pneumonia model. *J. Antimicrob. Chemother.* 63, 982–987. doi: 10.1093/jac/dkp056

Korotkov, K. V., Sandkvist, M., and Hol, W. G. (2012). The type II secretion system: biogenesis, molecular architecture and mechanism. *Nat. Rev. Microbiol.* 10, 336–351. doi: 10.1038/nrmicro2762

Kostoulias, X., Murray, G. L., Cerqueira, G. M., Kong, J. B., Bantun, F., Mylonakis, E., et al. (2015). Impact of a cross-kingdom signaling molecule of *Candida albicans* on *Acinetobacter baumannii* physiology. *Antimicrob. Agents Chemother.* 60, 161–167. doi: 10.1128/AAC.01540-15

Krizova, L., Poirel, L., Nordmann, P., and Nemec, A. (2013). TEM-1 β-lactamase as a source of resistance to sulbactam in clinical strains of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 68, 2786–2791. doi: 10.1093/jac/dkt275

Kulp, A., and Kuehn, M. J. (2010). Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* 64, 163–184. doi: 10.1146/annurev.micro.091208.073413

Kumar, M. (2016). Identification of a novel NDM variant, bla_{NDM-3}, from a multidrug-resistant *Acinetobacter baumannii*. *Infect. Control. Hosp. Epidemiol.* 37, 747–748. doi: 10.1017/ice.2016.66

Kuo, H. Y., Hsu, P. J., Chen, J. Y., Liao, P. C., Lu, C. W., Chen, C. H., et al. (2016). Clonal spread of $bla_{\text{OXA-72}}$-carrying *Acinetobacter baumannii* sequence type 512 in Taiwan. *Int. J. Antimicrob. Agents* 48, 111–113. doi: 10.1016/j.ijantimicag.2016.04.020

Kuo, H. Y., Yang, C. M., Lin, M. F., Cheng, W. L., Tien, N., and Liou, M. L. (2010). Distribution of $bla_{\text{OXA-carrying}}$ imipenem-resistant *Acinetobacter* spp. in 3 hospitals in Taiwan. *Diagn. Microbiol. Infect. Dis.* 66, 195–199. doi: 10.1016/j.diagmicrobio.2009.09.013

Kuo, L. C., Lai, C. C., Liao, C. H., Hsu, C. K., Chang, Y. L., Chang, C. Y., et al. (2007). Multidrug-resistant *Acinetobacter baumannii* bacteraemia:

clinical features, antimicrobial therapy and outcome. *Clin. Microbiol. Infect.* 13, 196–198. doi: 10.1111/j.1469-0691.2006.01601.x

Kuo, S. C., Chang, S. C., Wang, H. Y., Lai, J. F., Chen, P. C., Shiau, Y. R., et al. (2012). Emergence of extensively drug-resistant *Acinetobacter baumannii* complex over 10 years: nationwide data from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program. *BMC Infect Dis.* 12:200. doi: 10.1186/1471-2334-12-200

Kusradze, I., Karumidze, N., Rigvava, S., Dvalidze, T., Katsitadze, M., Amiranashvili, I., et al. (2016). Characterization and testing the efficiency of *Acinetobacter baumannii* phage *vB-GEC_Ab-M-G7* as an antibacterial agent. *Front. Microbiol.* 7:1590. doi: 10.3389/fmicb.2016.01590

Kwon, S. O., Gho, Y. S., Lee, J. C., and Kim, S. I. (2009). Proteome analysis of outer membrane vesicles from a clinical *Acinetobacter baumannii* isolate. *FEMS Microbiol. Lett.* 297, 150–156. doi: 10.1111/j.1574-6968.2009.01669.x

La Scola, B., Gundi, V. A., Khamis, A., and Raoult, D. (2006). Sequencing of the *rpoB* gene and flanking spacers for molecular identification of *Acinetobacter* species. *J. Clin. Microbiol.* 44, 827–832. doi: 10.1128/JCM.44.3.827-832.2006

Lee, C. H., Tang, Y. F., Su, L. H., Chien, C. C., and Liu, J. W. (2008). Antimicrobial effects of varied combinations of meropenem, sulbactam, and colistin on a multidrug-resistant *Acinetobacter baumannii* isolate that caused meningitis and bacteremia. *Microb. Drug Resist.* 14, 233–237. doi: 10.1089/mdr.2008.0840

Lee, C. R., Cho, I. H., Jeong, B. C., and Lee, S. H. (2013a). Strategies to minimize antibiotic resistance. *Int. J. Environ. Res. Public Health.* 10, 4274–4305. doi: 10.3390/ijerph10094274

Lee, C. R., Lee, J. H., Jeong, B. C., and Lee, S. H. (2013b). Lipid a biosynthesis of multidrug-resistant pathogens - a novel drug target. *Curr. Pharm. Des.* 19, 6534–6550. doi: 10.2174/13816128113199990494

Lee, C. R., Lee, J. H., Park, K. S., Jeong, B. C., and Lee, S. H. (2015). Quantitative proteomic view associated with resistance to clinically important antibiotics in Gram-positive bacteria: a systematic review. *Front. Microbiol.* 6:828. doi: 10.3389/fmicb.2015.00828

Lee, C. R., Lee, J. H., Park, K. S., Kim, Y. B., Jeong, B. C., and Lee, S. H. (2016). Global Dissemination of Carbapenemase-Producing *Klebsiella pneumoniae*: epidemiology, Genetic Context, Treatment Options, and Detection Methods. *Front. Microbiol.* 7:895. doi: 10.3389/fmicb.2016.00895

Lee, J. H., Choi, C. H., Kang, H. Y., Lee, J. Y., Kim, J., Lee, Y. C., et al. (2007). Differences in phenotypic and genotypic traits against antimicrobial agents between *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU. *J. Antimicrob. Chemother.* 59, 633–639. doi: 10.1093/jac/dkm007

Lee, J. H., Lee, J. J., Park, K. S., and Lee, S. H. (2015). Urgent need for β-lactam-β-lactamase inhibitors. *Lancet Infect. Dis.* 15, 876–877. doi: 10.1016/S1473-3099(15)00143-7

Lee, J. H., Park, K. S., Karim, A. M., Lee, C. R., and Lee, S. H. (2016). How to minimise antibiotic resistance. *Lancet Infect. Dis.* 16, 17–18. doi: 10.1016/S1473-3099(15)00467-3

Lee, J. S., Choi, C. H., Kim, J. W., and Lee, J. C. (2010). *Acinetobacter baumannii* outer membrane protein A induces dendritic cell death through mitochondrial targeting. *J. Microbiol.* 48, 387–392. doi: 10.1007/s12275-010-0155-1

Lee, K., Yum, J. H., Yong, D., Lee, H. M., Kim, H. D., Docquier, J. D., et al. (2005). Novel acquired metallo-β-lactamase gene, $bla_{SIM\text{-}1}$, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. *Antimicrob. Agents Chemother.* 49, 4485–4491. doi: 10.1128/AAC.49.11.4485-4491.2005

Lee, M. F., Peng, C. F., Hsu, H. J., and Chen, Y. H. (2008). Molecular characterisation of the metallo-β-lactamase genes in imipenem-resistant Gram-negative bacteria from a university hospital in southern Taiwan. *Int. J. Antimicrob. Agents* 32, 475–480. doi: 10.1016/j.ijantimicag.2008.07.009

Lee, Y., Kim, C. K., Lee, H., Jeong, S. H., Yong, D., and Lee, K. (2011). A novel insertion sequence, IS*Aba10*, inserted into ISAba1 adjacent to the $bla_{\text{OXA-23}}$ gene and disrupting the outer membrane protein gene *carO* in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 55, 361–363. doi: 10.1128/AAC.01672-09

Lee, Y. T., Tsao, S. M., and Hsueh, P. R. (2013). Clinical outcomes of tigecycline alone or in combination with other antimicrobial agents for the treatment of patients with healthcare-associated multidrug-resistant *Acinetobacter baumannii* infections. *Eur. J. Clin. Microbiol Infect Dis.* 32, 1211–1220. doi: 10.1007/s10096-013-1870-4

Lees-Miller, R. G., Iwashkiw, J. A., Scott, N. E., Seper, A., Vinogradov, E., Schild, S., et al. (2013). A common pathway for O-linked protein-glycosylation and synthesis of capsule in *Acinetobacter baumannii*. *Mol. Microbiol.* 89, 816–830. doi: 10.1111/mmi.12300

Lenhard, J. R., Bulitta, J. B., Connell, T. D., King-Lyons, N., Landersdorfer, C. B., Cheah, S. E., et al. (2016a). High-intensity meropenem combinations with polymyxin B: new strategies to overcome carbapenem resistance in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 72, 153–165. doi: 10.1093/jac/dkw355

Lenhard, J. R., Gall, J. S., Bulitta, J. B., Thamlikitkul, V., Landersdorfer, C. B., Forrest, A., et al. (2016b). Comparative pharmacodynamics of four different carbapenems in combination with polymyxin B against carbapenem-resistant *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents* 48, 719–724. doi: 10.1016/j.ijantimicag.2016.07.024

Li, L., Hassan, K. A., Brown, M. H., and Paulsen, I. T. (2016). Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 71, 1223–1232. doi: 10.1093/jac/dkv460

Li, X., Liu, L., Ji, J., Chen, Q., Hua, X., Jiang, Y., et al. (2015). Tigecycline resistance in *Acinetobacter baumannii* mediated by frameshift mutation in *plsC*, encoding 1-acyl-*sn*-glycerol-3-phosphate acyltransferase. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 625–631. doi: 10.1007/s10096-014-2272-y

Li, X. M., Choi, J. A., Choi, I. S., Kook, J. K., Chang, Y. H., Park, G., et al. (2016). Development and evaluation of species-specific PCR for detection of nine *Acinetobacter* species. *Ann. Clin. Lab. Sci.* 46, 270–278.

Li, X., Quan, J., Yang, Y., Ji, J., Liu, L., Fu, Y., et al. (2016). Abrp, a new gene, confers reduced susceptibility to tetracycline, glycylcine, chloramphenicol and fosfomycin classes in *Acinetobacter baumannii*. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 1371–1375. doi: 10.1007/s10096-016-2674-0

Li, Y., Guo, Q., Wang, P., Zhu, D., Ye, X., Wu, S., et al. (2015). Clonal dissemination of extensively drug-resistant *Acinetobacter baumannii* producing an OXA-23 β-lactamase at a teaching hospital in Shanghai, China. *J. Microbiol. Immunol. Infect.* 48, 101–108. doi: 10.1016/j.jmii.2014.04.005

Li, Z. T., Zhang, R. L., Bi, X. G., Xu, L., Fan, M., Xie, D., et al. (2015). Outer membrane vesicles isolated from two clinical *Acinetobacter baumannii* strains exhibit different toxicity and proteome characteristics. *Microb. Pathog.* 81, 46–52. doi: 10.1016/j.micpath.2015.03.009

Liang, W., Liu, X. F., Huang, J., Zhu, D. M., Li, J., and Zhang, J. (2011). Activities of colistin- and minocycline-based combinations against extensive drug resistant *Acinetobacter baumannii* isolates from intensive care unit patients. *BMC Infect. Dis.* 11:109. doi: 10.1186/1471-2334-11-109

Liao, Y. T., Kuo, S. C., Chiang, M. H., Lee, Y. T., Sung, W. C., Chen, Y. H., et al. (2015). *Acinetobacter baumannii* extracellular OXA-58 is primarily and selectively released via outer membrane vesicles after Sec-dependent periplasmic translocation. *Antimicrob. Agents Chemother.* 59, 7346–7354. doi: 10.1128/AAC.01343-15

Lin, L., Tan, B., Pantapalangkoor, P., Ho, T., Baquir, B., Tomaras, A., et al. (2012). Inhibition of LpxC protects mice from resistant *Acinetobacter baumannii* by modulating inflammation and enhancing phagocytosis. *MBio* 3:e00312-12. doi: 10.1128/mBio.00312-12

Lin, L., Tan, B., Pantapalangkoor, P., Ho, T., Hujer, A. M., Taracila, M. A., et al. (2013). *Acinetobacter baumannii* rOmpA vaccine dose alters immune polarization and immunodominant epitopes. *Vaccine* 31, 313–318. doi: 10.1016/j.vaccine.2012.11.008

Lin, M. F., Chang, K. C., Lan, C. Y., Chou, J., Kuo, J. W., Chang, C. K., et al. (2011a). Molecular epidemiology and antimicrobial resistance determinants of multidrug-resistant *Acinetobacter baumannii i*n five proximal hospitals in Taiwan. *Jpn. J. Infect. Dis.* 64, 222–227.

Lin, M. F., Chang, K. C., Yang, C. Y., Yang, C. M., Xiao, C. C., Kuo, H. Y., et al. (2010). Role of integrons in antimicrobial susceptibility patterns of *Acinetobacter baumannii*. *Jpn. J. Infect. Dis.* 63, 440–443.

Lin, M. F., Kuo, H. Y., Yeh, H. W., Yang, C. M., Sung, C. H., Tu, C. C., et al. (2011b). Emergence and dissemination of $bla_{\text{OXA-23}}$-carrying imipenem-resistant *Acinetobacter* sp. in a regional hospital in Taiwan. *J. Microbiol. Immunol. Infect.* 44, 39–44. doi: 10.1016/j.jmii.2011.01.008

Lin, M. F., and Lan, C. Y. (2014). Antimicrobial resistance in Acinetobacter baumannii: from bench to bedside. *World J. Clin. Cases.* 2, 787–814. doi: 10.12998/wjcc.v2.i12.787

Lin, M. F., Lin, Y. Y., and Lan, C. Y. (2015). The role of the two-component system BaeSR in disposing chemicals through regulating

transporter systems in *Acinetobacter baumannii*. *PLoS ONE* 10:e0132843. doi: 10.1371/journal.pone.0132843

Lin, M. F., Lin, Y. Y., Yeh, H. W., and Lan, C. Y. (2014). Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii adeAB* genes and its correlation with tigecycline susceptibility. *BMC Microbiol.* 14:119. doi: 10.1186/1471-2180-14-119

Lin, M. F., Liou, M. L., Tu, C. C., Yeh, H. W., and Lan, C. Y. (2013). Molecular epidemiology of integron-associated antimicrobial gene cassettes in the clinical isolates of *Acinetobacter baumannii* from northern Taiwan. *Ann. Lab. Med.* 33, 242–247. doi: 10.3343/alm.2013.33.4.242

Liou, M. L., Soo, P. C., Ling, S. R., Kuo, H. Y., Tang, C. Y., and Chang, K. C. (2014). The sensor kinase BfmS mediates virulence in *Acinetobacter baumannii*. *J. Microbiol. Immunol. Infect.* 47, 275–281. doi: 10.1016/j.jmii.2012.12.004

Liu, C. C., Kuo, H. Y., Tang, C. Y., Chang, K. C., and Liou, M. L. (2014). Prevalence and mapping of a plasmid encoding a type IV secretion system in *Acinetobacter baumannii*. *Genomics.* 104, 215–223. doi: 10.1016/j.ygeno.2014.07.011

Liu, D., Liu, Z. S., Hu, P., Cai, L., Fu, B. Q., Li, Y. S., et al. (2016). Characterization of surface antigen protein 1 (SurA1) from *Acinetobacter baumannii* and its role in virulence and fitness. *Vet. Microbiol.* 186, 126–138. doi: 10.1016/j.vetmic.2016.02.018

Liu, Q., Li, W., Feng, Y., and Tao, C. (2014). Efficacy and safety of polymyxins for the treatment of *Acinectobacter baumannii* infection: a systematic review and meta-analysis. *PLoS ONE* 9:e98091. doi: 10.1371/journal.pone.0098091

Liu, X., Zhao, M., Chen, Y., Bian, X., Li, Y., Shi, J., et al. (2016). Synergistic killing by meropenem and colistin combination of carbapenem-resistant *Acinetobacter baumannii* isolates from Chinese patients in an *in vitro* pharmacokinetic/pharmacodynamic model. *Int. J. Antimicrob. Agents* 48, 559–563. doi: 10.1016/j.ijantimicag.2016.07.018

Liu, Y., and Liu, X. (2015). Detection of AmpC β-lactamases in *Acinetobacter baumannii* in the Xuzhou region and analysis of drug resistance. *Exp. Ther. Med.* 10, 933–936. doi: 10.3892/etm.2015.2612

Lolans, K., Rice, T. W., Munoz-Price, L. S., and Quinn, J. P. (2006). Multicity outbreak of carbapenem-resistant *Acinetobacter baumannii* isolates producing the carbapenemase OXA-40. *Antimicrob. Agents Chemother.* 50, 2941–2945. doi: 10.1128/AAC.00116-06

Lomovskaya, O., and Lewis, K. (1992). Emr, an *Escherichia coli* locus for multidrug resistance. *Proc. Natl. Acad. Sci. U.S.A.* 89, 8938–8942.

Luke, N. R., Sauberan, S. L., Russo, T. A., Beanan, J. M., Olson, R., Loehfelm, T. W., et al. (2010). Identification and characterization of a glycosyltransferase involved in *Acinetobacter baumannii* lipopolysaccharide core biosynthesis. *Infect. Immun.* 78, 2017–2023. doi: 10.1128/IAI.00016-10

Luo, G., Lin, L., Ibrahim, A. S., Baquir, B., Pantapalangkoor, P., Bonomo, R. A., et al. (2012). Active and passive immunization protects against lethal, extreme drug resistant-*Acinetobacter baumannii* infection. *PLoS ONE* 7:e29446. doi: 10.1371/journal.pone.0029446

Lu, P. L., Doumith, M., Livermore, D. M., Chen, T. P., and Woodford, N. (2009). Diversity of carbapenem resistance mechanisms in *Acinetobacter baumannii* from a Taiwan hospital: spread of plasmid-borne OXA-72 carbapenemase. *J. Antimicrob. Chemother.* 63, 641–647. doi: 10.1093/jac/dkn553

Magnet, S., Courvalin, P., and Lambert, T. (2001). Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob. Agents Chemother.* 45, 3375–3380. doi: 10.1128/AAC.45.12.3375-3380.2001

Mak, J. K., Kim, M. J., Pham, J., Tapsall, J., and White, P. A. (2009). Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 63, 47–54. doi: 10.1093/jac/dkn454

Marchand, I., Damier-Piolle, L., Courvalin, P., and Lambert, T. (2004). Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. *Antimicrob. Agents Chemother.* 48, 3298–3304. doi: 10.1128/AAC.48.9.3298-3304.2004

Martinez-Guitian, M., Vazquez-Ucha, J. C., Odingo, J., Parish, T., Poza, M., Waite, R. D., et al. (2016). Synergy between colistin and the signal peptidase inhibitor MD3 is dependent on the mechanism of colistin resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 4375–4379. doi: 10.1128/AAC.00510-16

Martinez, T., Martinez, I., Vazquez, G. J., Aquino, E. E., and Robledo, I. E. (2016). Genetic environment of the KPC gene in *Acinetobacter baumannii* ST2 clone from Puerto Rico and genomic insights into its drug resistance. *J. Med. Microbiol.* 65, 784–792. doi: 10.1099/jmm.0.000289

McConnell, M. J., Actis, L., and Pachon, J. (2013). *Acinetobacter baumannii*: human infections, factors contributing to pathogenesis and animal models. *FEMS Microbiol. Rev.* 37, 130–155. doi: 10.1111/j.1574-6976.2012.00344.x

McConnell, M. J., Rumbo, C., Bou, G., and Pachon, J. (2011). Outer membrane vesicles as an acellular vaccine against *Acinetobacter baumannii*. *Vaccine* 29, 5705–5710. doi: 10.1016/j.vaccine.2011.06.001

McQueary, C. N., Kirkup, B. C., Si, Y., Barlow, M., Actis, L. A., Craft, D. W., et al. (2012). Extracellular stress and lipopolysaccharide modulate *Acinetobacter baumannii* surface-associated motility. *J. Microbiol.* 50, 434–443. doi: 10.1007/s12275-012-1555-1

Megeed, A. A., Hayssam, M. A., Salem, M. Z., El-Shikh, M. S., Talea, I. A., and Alogaibi, Y. A. (2016). Investigation of the virulence factors and molecular characterization of the clonal relations of multidrug-resistant *Acinetobacter baumannii* isolates. *J. AOAC Int.* doi: 10.5740/jaoacint.16-0139. [Epub ahead of print].

Mendes, R. E., Bell, J. M., Turnidge, J. D., Castanheira, M., and Jones, R. N. (2009). Emergence and widespread dissemination of OXA-23, -24/40 and -58 carbapenemases among *Acinetobacter* spp. in Asia-Pacific nations: report from the SENTRY Surveillance Program. *J. Antimicrob. Chemother.* 63, 55–59. doi: 10.1093/jac/dkn434

Mendes, R. E., Farrell, D. J., Sader, H. S., and Jones, R. N. (2010). Comprehensive assessment of tigecycline activity tested against a worldwide collection of *Acinetobacter* spp. (2005–2009). *Diagn. Microbiol. Infect. Dis.* 68, 307–311. doi: 10.1016/j.diagmicrobio.2010.07.003

Menegucci, T. C., Albiero, J., Migliorini, L. B., Alves, J. L., Viana, G. F., Mazucheli, J., et al. (2016). Strategies for the treatment of polymyxin B-resistant *Acinetobacter baumannii* infections. *Int. J. Antimicrob. Agents* 47, 380–385. doi: 10.1016/j.ijantimicag.2016.02.007

Merino, M., Acosta, J., Poza, M., Sanz, F., Beceiro, A., Chaves, F., et al. (2010). OXA-24 carbapenemase gene flanked by XerC/XerD-like recombination sites in different plasmids from different *Acinetobacter* species isolated during a nosocomial outbreak. *Antimicrob. Agents Chemother.* 54, 2724–2727. doi: 10.1128/AAC.01674-09

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D., Vinogradov, E., Seemann, T., et al. (2010). Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob. Agents Chemother.* 54, 4971–4977. doi: 10.1128/AAC.00834-10

Moland, E. S., Craft, D. W., Hong, S. G., Kim, S. Y., Hachmeister, L., Sayed, S. D., et al. (2008). *In vitro* activity of tigecycline against multidrug-resistant *Acinetobacter baumannii* and selection of tigecycline-amikacin synergy. *Antimicrob. Agents Chemother.* 52, 2940–2942. doi: 10.1128/AAC.01581-07

Montero, A., Ariza, J., Corbella, X., Domenech, A., Cabellos, C., Ayats, J., et al. (2004). Antibiotic combinations for serious infections caused by carbapenem-resistant *Acinetobacter baumannii* in a mouse pneumonia model. *J. Antimicrob. Chemother.* 54, 1085–1091. doi: 10.1093/jac/dkh485

Moon, D. C., Choi, C. H., Lee, J. H., Choi, C. W., Kim, H. Y., Park, J. S., et al. (2012). *Acinetobacter baumannii* outer membrane protein A modulates the biogenesis of outer membrane vesicles. *J. Microbiol.* 50, 155–160. doi: 10.1007/s12275-012-1589-4

Mosqueda, N., Espinal, P., Cosgaya, C., Viota, S., Plasensia, V., Alvarez-Lerma, F., et al. (2013). Globally expanding carbapenemase finally appears in Spain: nosocomial outbreak of *Acinetobacter baumannii* producing plasmid-encoded OXA-23 in Barcelona, Spain. *Antimicrob. Agents Chemother.* 57, 5155–5157. doi: 10.1128/AAC.01486-13

Motaouakkil, S., Charra, B., Hachimi, A., Nejmi, H., Benslama, A., Elmdaghri, N., et al. (2006). Colistin and rifampicin in the treatment of nosocomial infections from multiresistant *Acinetobacter baumannii*. *J. Infect.* 53, 274–278. doi: 10.1016/j.jinf.2005.11.019

Moubareck, C., Bremont, S., Conroy, M. C., Courvalin, P., and Lambert, T. (2009). GES-11, a novel integron-associated GES variant in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53, 3579–3581. doi: 10.1128/AAC.00072-09

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., et al. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312, 1526–1530. doi: 10.1126/science.1128393

Moya, B., Juan, C., Alberti, S., Perez, J. L., and Oliver, A. (2008). Benefit of having multiple *ampD* genes for acquiring β-lactam resistance without losing fitness

and virulence in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 52, 3694–3700. doi: 10.1128/AAC.00172-08

Mugnier, P. D., Poirel, L., Naas, T., and Nordmann, P. (2010). Worldwide dissemination of the bla_{OXA-23} carbapenemase gene of *Acinetobacter baumannii*. *Emerg. Infect. Dis.* 16, 35–40. doi: 10.3201/eid1601.090852

Mussi, M. A., Limansky, A. S., and Viale, A. M. (2005). Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter baumannii*: natural insertional inactivation of a gene encoding a member of a novel family of b-barrel outer membrane proteins. *Antimicrob. Agents Chemother.* 49, 1432–1440. doi: 10.1128/AAC.49.4.1432-1440.2005

Mussi, M. A., Relling, V. M., Limansky, A. S., and Viale, A. M. (2007). CarO, an *Acinetobacter baumannii* outer membrane protein involved in carbapenem resistance, is essential for L-ornithine uptake. *FEBS Lett.* 581, 5573–5578. doi: 10.1016/j.febslet.2007.10.063

Muthusamy, D., Sudhishnaa, S., and Boppe, A. (2016). *In vitro* activities of polymyxins and rifampicin against carbapenem resistant *Acinetobacter baumannii* at a tertiary care hospital from south India. *J. Clin.Diagn. Res.* 10, DC15–DC18. doi: 10.7860/JCDR/2016/19968.8535

Mutlu Yilmaz, E., Sunbul, M., Aksoy, A., Yilmaz, H., Guney, A. K., and Guvenc, T. (2012). Efficacy of tigecycline/colistin combination in a pneumonia model caused by extensively drug-resistant *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents* 40, 332–336. doi: 10.1016/j.ijantimicag.2012.06.003

Naas, T., Coignard, B., Carbonne, A., Blanckaert, K., Bajolet, O., Bernet, C., et al. (2006). VEB-1 Extended-spectrum β-lactamase-producing *Acinetobacter baumannii*, France. *Emerg. Infect. Dis.* 12, 1214–1222. doi: 10.3201/eid1208.051547

Naas, T., Levy, M., Hirschauer, C., Marchandin, H., and Nordmann, P. (2005). Outbreak of carbapenem-resistant *Acinetobacter baumannii* producing the carbapenemase OXA-23 in a tertiary care hospital of Papeete, French Polynesia. *J. Clin. Microbiol.* 43, 4826–4829. doi: 10.1128/JCM.43.9.4826-4829.2005

Naas, T., Namdari, F., Reglier-Poupet, H., Poyart, C., and Nordmann, P. (2007). Panresistant extended-spectrum β-lactamase SHV-5-producing *Acinetobacter baumannii* from New York City. *J. Antimicrob. Chemother.* 60, 1174–1176. doi: 10.1093/jac/dkm366

Nagano, N., Nagano, Y., Cordevant, C., Shibata, N., and Arakawa, Y. (2004). Nosocomial transmission of CTX-M-2 β-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward. *J. Clin. Microbiol.* 42, 3978–3984. doi: 10.1128/JCM.42.9.3978-3984.2004

Nairn, B. L., Lonergan, Z. R., Wang, J., Braymer, J. J., Zhang, Y., Calcutt, M. W., et al. (2016). The Response of *Acinetobacter baumannii* to Zinc Starvation. *Cell Host Microbe.* 19, 826–836. doi: 10.1016/j.chom.2016.05.007

Navia, M. M., Ruiz, J., and Vila, J. (2002). Characterization of an integron carrying a new class D β-lactamase (OXA-37) in *Acinetobacter baumannii*. *Microb. Drug Resist.* 8, 261–265. doi: 10.1089/10766290260469516

Navon-Venezia, S., Leavitt, A., and Carmeli, Y. (2007). High tigecycline resistance in multidrug-resistant *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 59, 772–774. doi: 10.1093/jac/dkm018

Nemec, A., Dolzani, L., Brisse, S., van den Broek, P., and Dijkshoorn, L. (2004). Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European *Acinetobacter baumannii* clones. *J. Med. Microbiol.* 53, 1233–1240. doi: 10.1099/jmm.0.45716-0

Nepka, M., Perivolioti, E., Kraniotaki, E., Politi, L., Tsakris, A., and Pournaras, S. (2016). *In vitro* bactericidal activity of trimethoprim-sulfamethoxazole alone and in combination with colistin, against carbapenem-resistant *Acinetobacter baumannii* clinical isolates. *Antimicrob. Agents Chemother.* doi: 10.1128/AAC.01082-16

Ni, W., Li, Y., Guan, J., Zhao, J., Cui, J., Wang, R., et al. (2016). Effects of efflux pump inhibitors on colistin resistance in multidrug-resistant Gram-negative gacteria. *Antimicrob. Agents Chemother.* 60, 3215–3218. doi: 10.1128/AAC.00248-16

Norton, M. D., Spilkia, A. J., and Godoy, V. G. (2013). Antibiotic resistance acquired through a DNA damage-inducible response in *Acinetobacter baumannii*. *J. Bacteriol.* 195, 1335–1345. doi: 10.1128/JB.02176-12

Nowak-Zaleska, A., Wieczor, M., Czub, J., Nierzwicki, L., Kotlowski, R., Mikucka, A., et al. (2016). Correlation between the number of Pro-Ala repeats in the EmrA homologue of *Acinetobacter baumannii* and resistance to netilmicin, tobramycin, imipenem and ceftazidime. *J. Glob. Antimicrob. Resist.* 7, 145–149. doi: 10.1016/j.jgar.2016.09.004

Ozbek, B., and Senturk, A. (2010). Postantibiotic effects of tigecycline, colistin sulfate, and levofloxacin alone or tigecycline-colistin sulfate and tigecycline-levofloxacin combinations against *Acinetobacter baumannii*. *Chemotherapy* 56, 466–471. doi: 10.1159/000321015

Pachon-Ibanez, M. E., Docobo-Perez, F., Lopez-Rojas, R., Dominguez-Herrera, J., Jimenez-Mejias, M. E., Garcia-Curiel, A., et al. (2010). Efficacy of rifampin and its combinations with imipenem, sulbactam, and colistin in experimental models of infection caused by imipenem-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 54, 1165–1172. doi: 10.1128/AAC.00367-09

Pachon-Ibanez, M. E., Jimenez-Mejias, M. E., Pichardo, C., Llanos, A. C., and Pachon, J. (2004). Activity of tigecycline (GAR-936) against *Acinetobacter baumannii* strains, including those resistant to imipenem. *Antimicrob. Agents Chemother.* 48, 4479–4481. doi: 10.1128/AAC.48.11.4479-4481.2004

Pailhories, H., Kempf, M., Belmonte, O., Joly-Guillou, M. L., and Eveillard, M. (2016). First case of OXA-24-producing *Acinetobacter baumannii* in cattle from Reunion Island, France. *Int. J. Antimicrob. Agents* 48, 763–764. doi: 10.1016/j.ijantimicag.2016.09.005

Pankuch, G. A., Lin, G., Seifert, H., and Appelbaum, P. C. (2008). Activity of meropenem with and without ciprofloxacin and colistin against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 52, 333–336. doi: 10.1128/AAC.00689-07

Pannek, S., Higgins, P. G., Steinke, P., Jonas, D., Akova, M., Bohnert, J. A., et al. (2006). Multidrug efflux inhibition in *Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-b-naphthylamide. *J. Antimicrob. Chemother.* 57, 970–974. doi: 10.1093/jac/dkl081

Pantopoulou, A., Giamarellos-Bourboulis, E. J., Raftogannis, M., Tsaganos, T., Dontas, I., Koutoukas, P., et al. (2007). Colistin offers prolonged survival in experimental infection by multidrug-resistant *Acinetobacter baumannii*: the significance of co-administration of rifampicin. *Int. J. Antimicrob. Agents* 29, 51–55. doi: 10.1016/j.ijantimicag.2006.09.009

Papa, A., Koulourida, V., and Souliou, E. (2009). Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* in a newly established Greek hospital. *Microb. Drug Resist.* 15, 257–260. doi: 10.1089/mdr.2009.0060

Park, Y. K., and Ko, K. S. (2015). Effect of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on killing *Acinetobacter baumannii* by colistin. *J. Microbiol.* 53, 53–59. doi: 10.1007/s12275-015-4498-5

Parra Millan, R., Jimenez Mejias, M. E., Sanchez Encinales, V., Ayerbe Algaba, R., Gutierrez Valencia, A., Pachon Ibanez, M. E., et al. (2016). Efficacy of lysophosphatidylcholine in combination with antimicrobial agents against *Acinetobacter baumannii* in experimental murine peritoneal sepsis and pneumonia models. *Antimicrob. Agents Chemother.* 60, 4464–4470. doi: 10.1128/AAC.02708-15

Pasteran, F., Rapoport, M., Petroni, A., Faccone, D., Corso, A., Galas, M., et al. (2006). Emergence of PER-2 and VEB-1a in *Acinetobacter baumannii* strains in the Americas. *Antimicrob. Agents Chemother.* 50, 3222–3224. doi: 10.1128/AAC.00284-06

Peck, K. R., Kim, M. J., Choi, J. Y., Kim, H. S., Kang, C. I., Cho, Y. K., et al. (2012). *In vitro* time-kill studies of antimicrobial agents against blood isolates of imipenem-resistant *Acinetobacter baumannii*, including colistin- or tigecycline-resistant isolates. *J. Med. Microbiol.* 61, 353–360. doi: 10.1099/jmm.0.036939-0

Peleg, A. Y., Adams, J., and Paterson, D. L. (2007). Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 51, 2065–2069. doi: 10.1128/AAC.01198-06

Peleg, A. Y., Seifert, H., and Paterson, D. L. (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbiol. Rev.* 21, 538–582. doi: 10.1128/CMR.00058-07

Pelletier, M. R., Casella, L. G., Jones, J. W., Adams, M. D., Zurawski, D. V., Hazlett, K. R., et al. (2013). Unique structural modifications are present in the lipopolysaccharide from colistin-resistant strains of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 57, 4831–4840. doi: 10.1128/AAC.00865-13

Penwell, W. F., Arivett, B. A., and Actis, L. A. (2012). The *Acinetobacter baumannii entA* gene located outside the acinetobactin cluster is critical for siderophore production, iron acquisition and virulence. *PLoS ONE* 7:e36493. doi: 10.1371/journal.pone.0036493

Perez, A., Merino, M., Rumbo-Feal, S., Alvarez-Fraga, L., Vallejo, J. A., Beceiro, A., et al. (2016). The FhaB/FhaC two-partner secretion system is involved

in adhesion of *Acinetobacter baumannii* AbH12O-A2 strain. *Virulence*. doi: 10.1080/21505594.2016.1262313. [Epub ahead of print].

Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N., and Bonomo, R. A. (2007). Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 51, 3471–3484. doi: 10.1128/AAC.01464-06

Petrosillo, N., Chinello, P., Proietti, M. F., Cecchini, L., Masala, M., Franchi, C., et al. (2005). Combined colistin and rifampicin therapy for carbapenem-resistant *Acinetobacter baumannii* infections: clinical outcome and adverse events. *Clin. Microbiol. Infect.* 11, 682–683. doi: 10.1111/j.1469-0691.2005.01198.x

Pfeifer, Y., Wilharm, G., Zander, E., Wichelhaus, T. A., Gottig, S., Hunfeld, K. P., et al. (2011). Molecular characterization of bla_{NDM-1} in an *Acinetobacter baumannii* strain isolated in Germany in 2007. *J. Antimicrob. Chemother.* 66, 1998–2001. doi: 10.1093/jac/dkr256

Phee, L. M., Betts, J. W., Bharathan, B., and Wareham, D. W. (2015). Colistin and fusidic acid, a novel potent synergistic combination for treatment of multidrug-resistant *Acinetobacter baumannii* infections. *Antimicrob. Agents Chemother.* 59, 4544–4550. doi: 10.1128/AAC.00753-15

Pichardo, C., Pachon-Ibanez, M. E., Docobo-Perez, F., Lopez-Rojas, R., Jimenez-Mejias, M. E., Garcia-Curiel, A., et al. (2010). Efficacy of tigecycline vs. imipenem in the treatment of experimental Acinetobacter baumannii murine pneumonia. *Eur. J. Clin. Microbiol. Infect. Dis.* 29, 527–531. doi: 10.1007/s10096-010-0890-6

Pires, J., Siriwardena, T. N., Stach, M., Tinguely, R., Kasraian, S., Luzzaro, F., et al. (2015). *In vitro* activity of the novel antimicrobial peptide dendrimer G3KL against multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 59, 7915–7918. doi: 10.1128/AAC.01853-15

Ploy, M. C., Giamarellou, H., Bourlioux, P., Courvalin, P., and Lambert, T. (1994). Detection of *aac(6′)*-I genes in amikacin-resistant *Acinetobacter* spp. by PCR. *Antimicrob. Agents Chemother.* 38, 2925–2928.

Poirel, L., Cabanne, L., Vahaboglu, H., and Nordmann, P. (2005a). Genetic environment and expression of the extended-spectrum β-lactamase bla_{PER-1} gene in gram-negative bacteria. *Antimicrob. Agents Chemother.* 49, 1708–1713. doi: 10.1128/AAC.49.5.1708-1713.2005

Poirel, L., Corvec, S., Rapoport, M., Mugnier, P., Petroni, A., Pasteran, F., et al. (2007). Identification of the novel narrow-spectrum β-lactamase SCO-1 in *Acinetobacter* spp. from Argentina. *Antimicrob. Agents Chemother.* 51, 2179–2184. doi: 10.1128/AAC.01600-06

Poirel, L., Mansour, W., Bouallegue, O., and Nordmann, P. (2008). Carbapenem-resistant *Acinetobacter baumannii* isolates from Tunisia producing the OXA-58-like carbapenem-hydrolyzing oxacillinase OXA-97. *Antimicrob. Agents Chemother.* 52, 1613–1617. doi: 10.1128/AAC.00978-07

Poirel, L., Marque, S., Heritier, C., Segonds, C., Chabanon, G., and Nordmann, P. (2005b). OXA-58, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 49, 202–208. doi: 10.1128/AAC.49.1.202-208.2005

Poirel, L., Mugnier, P. D., Toleman, M. A., Walsh, T. R., Rapoport, M. J., Petroni, A., et al. (2009). IS*CR2*, another vehicle for bla_{VEB} gene acquisition. *Antimicrob. Agents Chemother.* 53, 4940–4943. doi: 10.1128/AAC.00414-09

Pongpech, P., Amornnopparattanakul, S., Panapakdee, S., Fungwithaya, S., Nannha, P., Dhiraputra, C., et al. (2010). Antibacterial activity of carbapenem-based combinations againts multidrug-resistant *Acinetobacter baumannii*. *J. Med. Assoc. Thai.* 93, 161–171.

Potron, A., Munoz-Price, L. S., Nordmann, P., Cleary, T., and Poirel, L. (2011). Genetic features of CTX-M-15-producing *Acinetobacter baumannii* from Haiti. *Antimicrob. Agents Chemother.* 55, 5946–5948. doi: 10.1128/AAC.05124-11

Potron, A., Poirel, L., Croize, J., Chanteperdrix, V., and Nordmann, P. (2009). Genetic and biochemical characterization of the first extended-spectrum CARB-type b-lactamase, RTG-4, from *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53, 3010–3016. doi: 10.1128/AAC.01164-08

Pournaras, S., Markogiannakis, A., Ikonomidis, A., Kondyli, L., Bethimouti, K., Maniatis, A. N., et al. (2006). Outbreak of multiple clones of imipenem-resistant *Acinetobacter baumannii* isolates expressing OXA-58 carbapenemase in an intensive care unit. *J. Antimicrob. Chemother.* 57, 557–561. doi: 10.1093/jac/dkl004

Principe, L., Capone, A., Mazzarelli, A., D'Arezzo, S., Bordi, E., Di Caro, A., et al. (2013). *In vitro* activity of doripenem in combination with various antimicrobials against multidrug-resistant *Acinetobacter baumannii*: possible options for the treatment of complicated infection. *Microb. Drug Resist.* 19, 407–414. doi: 10.1089/mdr.2012.0250

Principe, L., D'Arezzo, S., Capone, A., Petrosillo, N., and Visca, P. (2009). *In vitro* activity of tigecycline in combination with various antimicrobials against multidrug resistant *Acinetobacter baumannii*. *Ann. Clin. Microbiol. Antimicrob.* 8:18. doi: 10.1186/1476-0711-8-18

Principe, L., Piazza, A., Giani, T., Bracco, S., Caltagirone, M. S., Arena, F., et al. (2014). Epidemic diffusion of OXA-23-producing *Acinetobacter baumannii* isolates in Italy: results of the first cross-sectional countrywide survey. *J. Clin. Microbiol.* 52, 3004–3010. doi: 10.1128/JCM.00291-14

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., et al. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1528–1533. doi: 10.1073/pnas.0510322103

Qi, C., Malczynski, M., Parker, M., and Scheetz, M. H. (2008). Characterization of genetic diversity of carbapenem-resistant *Acinetobacter baumannii* clinical strains collected from 2004 to 2007. *J. Clin. Microbiol.* 46, 1106–1109. doi: 10.1128/JCM.01877-07

Quale, J., Bratu, S., Landman, D., and Heddurshetti, R. (2003). Molecular epidemiology and mechanisms of carbapenem resistance in *Acinetobacter baumannii* endemic in New York City. *Clin. Infect. Dis.* 37, 214–220. doi: 10.1086/375821

Quinteira, S., Grosso, F., Ramos, H., and Peixe, L. (2007). Molecular epidemiology of imipenem-resistant *Acinetobacter haemolyticus* and *Acinetobacter baumannii* isolates carrying plasmid-mediated OXA-40 from a Portuguese hospital. *Antimicrob. Agents Chemother.* 51, 3465–3466. doi: 10.1128/AAC.00267-07

Rafailidis, P. I., Ioannidou, E. N., and Falagas, M. E. (2007). Ampicillin/sulbactam: current status in severe bacterial infections. *Drugs* 67, 1829–1849. doi: 10.2165/00003495-200767130-00003

Rajamohan, G., Srinivasan, V. B., and Gebreyes, W. A. (2010). Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 65, 1919–1925. doi: 10.1093/jac/dkq195

Rakin, A., Schneider, L., and Podladchikova, O. (2012). Hunger for iron: the alternative siderophore iron scavenging systems in highly virulent *Yersinia*. *Front. Cell. Infect. Microbiol.* 2:151. doi: 10.3389/fcimb.2012.00151

Ramirez, M. S., Don, M., Merkier, A. K., Bistue, A. J., Zorreguieta, A., Centron, D., et al. (2010a). Naturally competent *Acinetobacter baumannii* clinical isolate as a convenient model for genetic studies. *J. Clin. Microbiol.* 48, 1488–1490. doi: 10.1128/JCM.01264-09

Ramirez, M. S., Pineiro, S., Argentinian Integron Study Group, and Centron, D. (2010b). Novel insights about class 2 integrons from experimental and genomic epidemiology. *Antimicrob. Agents Chemother.* 54, 699–706. doi: 10.1128/AAC.01392-08

Rao, G. G., Ly, N. S., Bulitta, J. B., Soon, R. L., San Roman, M. D., Holden, P. N., et al. (2016a). Polymyxin B in combination with doripenem against heteroresistant *Acinetobacter baumannii:* pharmacodynamics of new dosing strategies. *J. Antimicrob. Chemother.* 71, 3148–3156. doi: 10.1093/jac/dkw293

Rao, G. G., Ly, N. S., Diep, J., Forrest, A., Bulitta, J. B., Holden, P. N., et al. (2016b). Combinatorial pharmacodynamics of polymyxin B and tigecycline against heteroresistant *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents* 48, 331–336. doi: 10.1016/j.ijantimicag.2016.06.006

Ravasi, P., Limansky, A. S., Rodriguez, R. E., Viale, A. M., and Mussi, M. A. (2011). IS*Aba825*, a functional insertion sequence modulating genomic plasticity and bla_{OXA-58} expression in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 55, 917–920. doi: 10.1128/AAC.00491-10

Reid, G., Food and Agricultural Organization of the United Nation and the WHO (2005). The importance of guidelines in the development and application of probiotics. *Curr. Pharm. Des.* 11, 11–16. doi: 10.2174/1381612053382395

Repizo, G. D., Gagne, S., Foucault-Grunenwald, M. L., Borges, V., Charpentier, X., Limansky, A. S., et al. (2015). Differential Role of the T6SS in *Acinetobacter baumannii* Virulence. *PLoS ONE* 10:e0138265. doi: 10.1371/journal.pone.0138265

Ribera, A., Roca, I., Ruiz, J., Gibert, I., and Vila, J. (2003a). Partial characterization of a transposon containing the *tet(A)* determinant in a clinical

isolate of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 52, 477–480. doi: 10.1093/jac/dkg344

Ribera, A., Ruiz, J., and Vila, J. (2003b). Presence of the Tet M determinant in a clinical isolate of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother*. 47, 2310–2312. doi: 10.1128/AAC.47.7.2310-2312.2003

Riccio, M. L., Franceschini, N., Boschi, L., Caravelli, B., Cornaglia, G., Fontana, R., et al. (2000). Characterization of the metallo-β-lactamase determinant of *Acinetobacter baumannii* AC-54/97 reveals the existence of bla_{IMP} allelic variants carried by gene cassettes of different phylogeny. *Antimicrob. Agents Chemother*. 44, 1229–1235. doi: 10.1128/AAC.44.5.1229-1235.2000

Ritchie, D. J., and Garavaglia-Wilson, A. (2014). A review of intravenous minocycline for treatment of multidrug-resistant *Acinetobacter* infections. *Clin. Infect. Dis.* 59 (Suppl. 6), S374–S380. doi: 10.1093/cid/ciu613

Robledo, I. E., Aquino, E. E., Sante, M. I., Santana, J. L., Otero, D. M., Leon, C. F., et al. (2010). Detection of KPC in *Acinetobacter* spp. in Puerto Rico. *Antimicrob. Agents Chemother.* 54, 1354–1357. doi: 10.1128/AAC.00899-09

Roca, I., Marti, S., Espinal, P., Martinez, P., Gibert, I., and Vila, J. (2009). CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53, 4013–4014. doi: 10.1128/AAC.00584-09

Rodriguez-Bano, J., Marti, S., Soto, S., Fernandez-Cuenca, F., Cisneros, J. M., Pachon, J., et al. (2008). Biofilm formation in *Acinetobacter baumannii:* associated features and clinical implications. *Clin. Microbiol. Infect.* 14, 276–278. doi: 10.1111/j.1469-0691.2007.01916.x

Rodriguez, C. H., De Ambrosio, A., Bajuk, M., Spinozzi, M., Nastro, M., Bombicino, K., et al. (2010). *In vitro* antimicrobials activity against endemic *Acinetobacter baumannii* multiresistant clones. *J. Infect. Dev. Ctries*. 4, 164–167. doi: 10.3855/jidc.604

Rodriguez, C. H., Nastro, M., Vay, C., and Famiglietti, A. (2015). *In vitro* activity of minocycline alone or in combination in multidrug-resistant *Acinetobacter baumannii* isolates. *J. Med. Microbiol.* 64, 1196–1200. doi: 10.1099/jmm.0.000147

Rodriguez-Rubio, L., Chang, W. L., Gutierrez, D., Lavigne, R., Martinez, B., Rodriguez, A., et al. (2016). 'Artilysation' of endolysin lambdaSa2lys strongly improves its enzymatic and antibacterial activity against streptococci. *Sci. Rep.* 6:35382. doi: 10.1038/srep35382

Rosenfeld, N., Bouchier, C., Courvalin, P., and Perichon, B. (2012). Expression of the resistance-nodulation-cell division pump AdeIJK in *Acinetobacter baumannii* is regulated by AdeN, a TetR-type regulator. *Antimicrob. Agents Chemother.* 56, 2504–2510. doi: 10.1128/AAC.06422-11

Ruiz, F. M., Santillana, E., Spinola-Amilibia, M., Torreira, E., Culebras, E., and Romero, A. (2015). Crystal Structure of Hcp from *Acinetobacter baumannii:* a Component of the Type VI Secretion System. *PLoS ONE* 10:e0129691. doi: 10.1371/journal.pone.0129691

Ruiz, M., Marti, S., Fernandez-Cuenca, F., Pascual, A., and Vila, J. (2007). High prevalence of carbapenem-hydrolysing oxacillinases in epidemiologically related and unrelated *Acinetobacter baumannii* clinical isolates in Spain. *Clin. Microbiol. Infect.* 13, 1192–1198. doi: 10.1111/j.1469-0691.2007.01825.x

Rumbo, C., Fernandez-Moreira, E., Merino, M., Poza, M., Mendez, J. A., Soares, N. C., et al. (2011). Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 55, 3084–3090. doi: 10.1128/AAC.00929-10

Rumbo, C., Tomas, M., Fernandez Moreira, E., Soares, N. C., Carvajal, M., Santillana, E., et al. (2014). The *Acinetobacter baumannii* Omp33-36 porin is a virulence factor that induces apoptosis and modulates autophagy in human cells. *Infect. Immun.* 82, 4666–4680. doi: 10.1128/IAI.02034-14

Rumbo, C., Vallejo, J. A., Cabral, M. P., Martinez-Guitian, M., Perez, A., Beceiro, A., et al. (2016). Assessment of antivirulence activity of several D-amino acids against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 71, 3473–3481. doi: 10.1093/jac/dkw342

Russo, T. A., Beanan, J. M., Olson, R., MacDonald, U., Cox, A. D., St. Michael, F., et al. (2013). The K1 capsular polysaccharide from *Acinetobacter baumannii* is a potential therapeutic target via passive immunization. *Infect. Immun.* 81, 915–922. doi: 10.1128/IAI.01184-12

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., MacDonald, U., et al. (2010). The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. *Infect. Immun.* 78, 3993–4000. doi: 10.1128/IAI.00366-10

Russo, T. A., MacDonald, U., Beanan, J. M., Olson, R., MacDonald, I. J., Sauberan, S. L., et al. (2009). Penicillin-binding protein 7/8 contributes to the survival of *Acinetobacter baumannii in vitro* and *in vivo*. *J. Infect. Dis.* 199, 513–521. doi: 10.1086/596317

Russo, T. A., Manohar, A., Beanan, J. M., Olson, R., MacDonald, U., Graham, J., et al. (2016). The response regulator BfmR is a potential drug target for *Acinetobacter baumannii*. *mSphere* 1:e00082-16. doi: 10.1128/mSphere.00082-16

Ruzin, A., Keeney, D., and Bradford, P. A. (2007). AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. *J. Antimicrob. Chemother.* 59, 1001–1004. doi: 10.1093/jac/dkm058

Saha, R., Saha, N., Donofrio, R. S., and Bestervelt, L. L. (2013). Microbial siderophores: a mini review. *J. Basic Microbiol.* 53, 303–317. doi: 10.1002/jobm.201100552

Sahly, H., Navon-Venezia, S., Roesler, L., Hay, A., Carmeli, Y., Podschun, R., et al. (2008). Extended-spectrum β-lactamase production is associated with an increase in cell invasion and expression of fimbrial adhesins in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* 52, 3029–3034. doi: 10.1128/AAC.00010-08

Sakoulas, G., Rose, W., Berti, A., Olson, J., Mungia, J., Nonejuie, P., et al. (2016). Classical β-lactamase inhibitors potentiate the activity of daptomycin against methicillin-resistant *Staphylococcus aureus* and colistin against *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 61:e01745-16. doi: 10.1128/AAC.01745-16

Sandri, A. M., Landersdorfer, C. B., Jacob, J., Boniatti, M. M., Dalarosa, M. G., Falci, D. R., et al. (2013). Population pharmacokinetics of intravenous polymyxin B in critically ill patients: implications for selection of dosage regimens. *Clin. Infect. Dis.* 57, 524–531. doi: 10.1093/cid/cit334

Santimaleeworagun, W., Wongpoowarak, P., Chayakul, P., Pattharachayakul, S., Tansakul, P., and Garey, K. W. (2011). *In vitro* activity of colistin or sulbactam in combination with fosfomycin or imipenem against clinical isolates of carbapenem-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemases. *Southeast Asian J. Trop. Med. Public Health*. 42, 890–900.

Schroder, W., Goerke, C., and Wolz, C. (2013). Opposing effects of aminocoumarins and fluoroquinolones on the SOS response and adaptability in *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 68, 529–538. doi: 10.1093/jac/dks456

Sechi, L. A., Karadenizli, A., Deriu, A., Zanetti, S., Kolayli, F., Balikci, E., et al. (2004). PER-1 type β-lactamase production in *Acinetobacter baumannii* is related to cell adhesion. *Med. Sci. Monit*. 10, BR180–BR184.

Segal, H., Nelson, E. C., and Elisha, B. G. (2004). Genetic environment and transcription of *ampC* in an *Acinetobacter baumannii* clinical isolate. *Antimicrob. Agents Chemother.* 48, 612–614. doi: 10.1128/AAC.48.2.612-614.2004

Sharma, A., Sharma, R., Bhattacharyya, T., Bhando, T., and Pathania, R. (2016). Fosfomycin resistance in *Acinetobacter baumannii* is mediated by efflux through a major facilitator superfamily (MFS) transporter-AbaF. *J. Antimicrob. Chemother.* 72, 68–74. doi: 10.1093/jac/dkw382

Sheng, W. H., Wang, J. T., Li, S. Y., Lin, Y. C., Cheng, A., Chen, Y. C., et al. (2011). Comparative *in vitro* antimicrobial susceptibilities and synergistic activities of antimicrobial combinations against carbapenem-resistant *Acinetobacter* species: *Acinetobacter baumannii* versus *Acinetobacter* genospecies 3 and 13TU. *Diagn. Microbiol. Infect. Dis.* 70, 380–386. doi: 10.1016/j.diagmicrobio.2011.03.003

Shin, B., and Park, W. (2015). Synergistic effect of oleanolic acid on aminoglycoside antibiotics against *Acinetobacter baumannii*. *PLoS ONE* 10:e0137751. doi: 10.1371/journal.pone.0137751

Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., and Leiman, P. G. (2013). PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature* 500, 350–353. doi: 10.1038/nature12453

Siroy, A., Molle, V., Lemaitre-Guillier, C., Vallenet, D., Pestel-Caron, M., Cozzone, A. J., et al. (2005). Channel formation by CarO, the carbapenem resistance-associated outer membrane protein of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 49, 4876–4883. doi: 10.1128/AAC.49.12.4876-4883.2005

Skalweit, M. J., and Li, M. (2016). Bulgecin A as a β-lactam enhancer for carbapenem-resistant *Pseudomonas aeruginosa* and carbapenem-resistant *Acinetobacter baumannii* clinical isolates containing various resistance

mechanisms. *Drug Des. Devel. Ther.* 10, 3013–3020. doi: 10.2147/DDDT.S110193

Smani, Y., Dominguez-Herrera, J., and Pachon, J. (2013). Association of the outer membrane protein Omp33 with fitness and virulence of *Acinetobacter baumannii*. *J. Infect. Dis.* 208, 1561–1570. doi: 10.1093/infdis/jit386

Smani, Y., Fabrega, A., Roca, I., Sanchez-Encinales, V., Vila, J., and Pachon, J. (2014). Role of OmpA in the multidrug resistance phenotype of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 58, 1806–1808. doi: 10.1128/AAC.02101-13

Smani, Y., McConnell, M. J., and Pachon, J. (2012). Role of fibronectin in the adhesion of *Acinetobacter baumannii* to host cells. *PLoS ONE* 7:e33073. doi: 10.1371/journal.pone.0033073

Smith, S. G., Mahon, V., Lambert, M. A., and Fagan, R. P. (2007). A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiol. Lett.* 273, 1–11. doi: 10.1111/j.1574-6968.2007.00778.x

Smolyakov, R., Borer, A., Riesenberg, K., Schlaeffer, F., Alkan, M., Porath, A., et al. (2003). Nosocomial multi-drug resistant *Acinetobacter baumannii* bloodstream infection: risk factors and outcome with ampicillin-sulbactam treatment. *J. Hosp. Infect.* 54, 32–38. doi: 10.1016/S0195-6701(03)00046-X

Songer, J. G. (1997). Bacterial phospholipases and their role in virulence. *Trends Microbiol.* 5, 156–161. doi: 10.1016/S0966-842X(97)01005-6

Song, J. Y., Lee, J., Heo, J. Y., Noh, J. Y., Kim, W. J., Cheong, H. J., et al. (2008). Colistin and rifampicin combination in the treatment of ventilator-associated pneumonia caused by carbapenem-resistant *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents* 32, 281–284. doi: 10.1016/j.ijantimicag.2008.04.013

Srinivasan, V. B., Rajamohan, G., and Gebreyes, W. A. (2009). Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53, 5312–5316. doi: 10.1128/AAC.00748-09

Srinivasan, V. B., Venkataramaiah, M., Mondal, A., and Rajamohan, G. (2015). Functional characterization of AbeD, an RND-type membrane transporter in antimicrobial resistance in *Acinetobacter baumannii*. *PLoS ONE* 10:e0141314. doi: 10.1371/journal.pone.0141314

Stahl, J., Bergmann, H., Gottig, S., Ebersberger, I., and Averhoff, B. (2015). *Acinetobacter baumannii* virulence is mediated by the concerted action of three phospholipases D. *PLoS ONE* 10:e0138360. doi: 10.1371/journal.pone.0138360

Stoeva, T., Higgins, P. G., Bojkova, K., and Seifert, H. (2008). Clonal spread of carbapenem-resistant OXA-23-positive *Acinetobacter baumannii* in a Bulgarian university hospital. *Clin. Microbiol. Infect.* 14, 723–727. doi: 10.1111/j.1469-0691.2008.02018.x

Su, C. H., Wang, J. T., Hsiung, C. A., Chien, L. J., Chi, C. L., Yu, H. T., et al. (2012). Increase of carbapenem-resistant *Acinetobacter baumannii* infection in acute care hospitals in Taiwan: association with hospital antimicrobial usage. *PLoS ONE* 7:e37788. doi: 10.1371/journal.pone.0037788

Sugawara, E., and Nikaido, H. (2012). OmpA is the principal nonspecific slow porin of *Acinetobacter baumannii*. *J. Bacteriol.* 194, 4089–4096. doi: 10.1128/JB.00435-12

Sun, J. R., Jeng, W. Y., Perng, C. L., Yang, Y. S., Soo, P. C., Chiang, Y. S., et al. (2016). Single amino acid substitution Gly186Val in AdeS restores tigecycline susceptibility of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 71, 1488–1492. doi: 10.1093/jac/dkw002

Sun, J. R., Perng, C. L., Chan, M. C., Morita, Y., Lin, J. C., Su, C. M., et al. (2012). A truncated AdeS kinase protein generated by IS*Aba1* insertion correlates with tigecycline resistance in *Acinetobacter baumannii*. *PLoS ONE* 7:e49534. doi: 10.1371/journal.pone.0049534

Su, X. Z., Chen, J., Mizushima, T., Kuroda, T., and Tsuchiya, T. (2005). AbeM, an H^+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob. Agents Chemother.* 49, 4362–4364. doi: 10.1128/AAC.49.10.4362-4364.2005

Tada, T., Miyoshi-Akiyama, T., Shimada, K., Shimojima, M., and Kirikae, T. (2014). Dissemination of 16S rRNA methylase ArmA-producing *Acinetobacter baumannii* and emergence of OXA-72 carbapenemase coproducers in Japan. *Antimicrob. Agents Chemother.* 58, 2916–2920. doi: 10.1128/AAC.01212-13

Taitt, C. R., Leski, T. A., Stockelman, M. G., Craft, D. W., Zurawski, D. V., Kirkup, B. C., et al. (2014). Antimicrobial resistance determinants in *Acinetobacter baumannii* isolates taken from military treatment facilities. *Antimicrob. Agents Chemother.* 58, 767–781. doi: 10.1128/AAC.01897-13

Thandar, M., Lood, R., Winer, B. Y., Deutsch, D. R., Euler, C. W., and Fischetti, V. A. (2016). Novel engineered peptides of a phage lysin as effective antimicrobials against multidrug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 2671–2679. doi: 10.1128/AAC.02972-15

Thummeepak, R., Kitti, T., Kunthalert, D., and Sitthisak, S. (2016). Enhanced antibacterial activity of *Acinetobacter baumannii* bacteriophage OABP-01 endolysin (LysABP-01) in combination with colistin. *Front. Microbiol.* 7:1402. doi: 10.3389/fmicb.2016.01402

Timurkaynak, F., Can, F., Azap, O. K., Demirbilek, M., Arslan, H., and Karaman, S. O. (2006). *In vitro* activities of non-traditional antimicrobials alone or in combination against multidrug-resistant strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from intensive care units. *Int. J. Antimicrob. Agents* 27, 224–228. doi: 10.1016/j.ijantimicag.2005.10.012

Tipton, K. A., and Rather, P. N. (2016). An ompR/envZ two-component system ortholog regulates phase variation, osmotic tolerance, motility, and virulence in *Acinetobacter baumannii* strain AB5075. *J. Bacteriol.* doi: 10.1128/JB.00705-16. [Epub ahead of print].

Tognim, M. C., Gales, A. C., Penteado, A. P., Silbert, S., and Sader, H. S. (2006). Dissemination of IMP-1 metallo- beta -lactamase-producing *Acinetobacter* species in a Brazilian teaching hospital. *Infect. Control Hosp. Epidemiol.* 27, 742–747. doi: 10.1086/504356

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., and Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii:* involvement of a novel chaperone-usher pili assembly system. *Microbiology* 149, 3473–3484. doi: 10.1099/mic.0.26541-0

Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. A., and Actis, L. A. (2008). Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology* 154, 3398–3409. doi: 10.1099/mic.0.2008/019471-0

Touchon, M., Cury, J., Yoon, E. J., Krizova, L., Cerqueira, G. C., Murphy, C., et al. (2014). The genomic diversification of the whole *Acinetobacter* genus: origins, mechanisms, and consequences. *Genome Biol. Evol.* 6, 2866–2882. doi: 10.1093/gbe/evu225

Traglia, G. M., Chua, K., Centron, D., Tolmasky, M. E., and Ramirez, M. S. (2014). Whole-genome sequence analysis of the naturally competent *Acinetobacter baumannii* clinical isolate A118. *Genome Biol. Evol.* 6, 2235–2239. doi: 10.1093/gbe/evu176

Traglia, G. M., Quinn, B., Schramm, S. T., Soler-Bistue, A., and Ramirez, M. S. (2016). Serum Albumin and Ca^{2+} Are Natural Competence Inducers in the Human Pathogen *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 4920–4929. doi: 10.1128/AAC.00529-16

Trebosc, V., Gartenmann, S., Royet, K., Manfredi, P., Totzl, M., Schellhorn, B., et al. (2016). A novel genome-editing platform for drug-resistant *Acinetobacter baumannii* reveals an AdeR-unrelated tigecycline resistance mechanism. *Antimicrob. Agents Chemother.* 60, 7263–7271. doi: 10.1128/AAC.01275-16

Tsakris, A., Ikonomidis, A., Poulou, A., Spanakis, N., Vrizas, D., Diomidous, M., et al. (2008). Clusters of imipenem-resistant *Acinetobacter baumannii* clones producing different carbapenemases in an intensive care unit. *Clin. Microbiol. Infect.* 14, 588–594. doi: 10.1111/j.1469-0691.2008.01996.x

Tsakris, A., Ikonomidis, A., Pournaras, S., Tzouvelekis, L. S., Sofianou, D., Legakis, N. J., et al. (2006). VIM-1 metallo-β-lactamase in *Acinetobacter baumannii*. *Emerg. Infect. Dis.* 12, 981–983. doi: 10.3201/eid1206.051097

Tsakris, A., Ikonomidis, A., Spanakis, N., Pournaras, S., and Bethimouti, K. (2007). Identification of a novel $bla_{\text{OXA-51}}$ variant, $bla_{\text{OXA-92}}$, from a clinical isolate of *Acinetobacter baumannii*. *Clin. Microbiol. Infect.* 13, 348–349. doi: 10.1111/j.1469-0691.2006.01598.x

Tsuji, B. T., Landersdorfer, C. B., Lenhard, J. R., Cheah, S. E., Thamlikitkul, V., Rao, G. G., et al. (2016). Paradoxical effect of polymyxin B: high drug exposure amplifies resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 3913–3920. doi: 10.1128/AAC.02831-15

Turner, P. J., Greenhalgh, J. M., and MYSTIC Study Group (Europe) (2003). The activity of meropenem and comparators against *Acinetobacter* strains isolated from European hospitals, 1997–2000. *Clin. Microbiol. Infect.* 9, 563–567. doi: 10.1046/j.1469-0691.2003.00591.x

Turton, J. F., Ward, M. E., Woodford, N., Kaufmann, M. E., Pike, R., Livermore, D. M., et al. (2006a). The role of IS*Aba1* in expression of OXA

carbapenemase genes in *Acinetobacter baumannii*. *FEMS Microbiol. Lett.* 258, 72–77. doi: 10.1111/j.1574-6968.2006.00195.x

Turton, J. F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E., and Pitt, T. L. (2006b). Identification of *Acinetobacter baumannii* by detection of the $bla_{\text{OXA-51-like}}$ carbapenemase gene intrinsic to this species. *J. Clin Microbiol.* 44, 2974–2976. doi: 10.1128/JCM.01021-06

Umland, T. C., Schultz, L. W., MacDonald, U., Beanan, J. M., Olson, R., and Russo, T. A. (2012). *In vivo*-validated essential genes identified in *Acinetobacter baumannii* by using human ascites overlap poorly with essential genes detected on laboratory media. *MBio*. 3:e00113-12. doi: 10.1128/mBio.00113-12

Vahaboglu, H., Budak, F., Kasap, M., Gacar, G., Torol, S., Karadenizli, A., et al. (2006). High prevalence of OXA-51-type class D β-lactamases among ceftazidime-resistant clinical isolates of *Acinetobacter* spp.: co-existence with OXA-58 in multiple centres. *J. Antimicrob. Chemother.* 58, 537–542. doi: 10.1093/jac/dkl273

Valenzuela, J. K., Thomas, L., Partridge, S. R., van der Reijden, T., Dijkshoorn, L., and Iredell, J. (2007). Horizontal gene transfer in a polyclonal outbreak of carbapenem-resistant *Acinetobacter baumannii*. *J. Clin. Microbiol.* 45, 453–460. doi: 10.1128/JCM.01971-06

Vijayakumar, S., Gopi, R., Gunasekaran, P., Bharathy, M., Walia, K., Anandan, S., et al. (2016). Molecular characterization of invasive carbapenem-resistant *Acinetobacter baumannii* from a tertiary care hospital in south India. *Infect. Dis. Ther.* 5, 379–387. doi: 10.1007/s40121-016-0125-y

Vilacoba, E., Almuzara, M., Gulone, L., Traglia, G. M., Figueroa, S. A., Sly, G., et al. (2013). Emergence and spread of plasmid-borne *tet(B)*::IS*CR2* in minocycline-resistant *Acinetobacter baumannii* isolates. *Antimicrob. Agents Chemother.* 57, 651–654. doi: 10.1128/AAC.01751-12

Vila, J., Navia, M., Ruiz, J., and Casals, C. (1997). Cloning and nucleotide sequence analysis of a gene encoding an OXA-derived β-lactamase in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother*. 41, 2757–2759.

Vila, J., Ruiz, J., Goni, P., Marcos, A., and Jimenez de Anta, T. (1995). Mutation in the *gyrA* gene of quinolone-resistant clinical isolates of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother*. 39, 1201–1203.

Vincent, J. L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C. D., et al. (2009). International study of the prevalence and outcomes of infection in intensive care units. *JAMA* 302, 2323–2329. doi: 10.1001/jama.2009.1754

Voulgari, E., Politi, L., Pitiriga, V., Dendrinos, J., Poulou, A., Georgiadis, G., et al. (2016). First report of an NDM-1 metallo-β-lactamase-producing *Acinetobacter baumannii* clinical isolate in Greece. *Int. J. Antimicrob. Agents* 48, 761–762. doi: 10.1016/j.ijantimicag.2016.09.006

Wang, H., Guo, P., Sun, H., Wang, H., Yang, Q., Chen, M., et al. (2007). Molecular epidemiology of clinical isolates of carbapenem-resistant *Acinetobacter* spp. from Chinese hospitals. Antimicrob. *Agents Chemother.* 51, 4022–4028. doi: 10.1128/AAC.01259-06

Wang, N., Ozer, E. A., Mandel, M. J., and Hauser, A. R. (2014). Genome-wide identification of *Acinetobacter baumannii* genes necessary for persistence in the lung. *MBio* 5, e01163–14. doi: 10.1128/mBio.01163-14

Wareham, D. W., Gordon, N. C., and Hornsey, M. (2011). *In vitro* activity of teicoplanin combined with colistin versus multidrug-resistant strains of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 66, 1047–1051. doi: 10.1093/jac/dkr069

Weber, B. S., Harding, C. M., and Feldman, M. F. (2015a). Pathogenic Acinetobacter: from the Cell Surface to Infinity and Beyond. *J. Bacteriol.* 198, 880–887. doi: 10.1128/JB.00906-15

Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S., and Feldman, M. F. (2015b). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proc. Natl.Acad. Sci. U.S.A.* 112, 9442–9447. doi: 10.1073/pnas.1502966112

Weber, B. S., Miyata, S. T., Iwashkiw, J. A., Mortensen, B. L., Skaar, E. P., Pukatzki, S., et al. (2013). Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. *PLoS ONE* 8:e55142. doi: 10.1371/journal.pone.0055142

Wormser, G. P., Keusch, G. T., and Heel, R. C. (1982). Co-trimoxazole (trimethoprim-sulfamethoxazole): an updated review of its antibacterial activity and clinical efficacy. *Drugs* 24, 459–518.

Wright, M. S., Haft, D. H., Harkins, D. M., Perez, F., Hujer, K. M., Bajaksouzian, S., et al. (2014). New insights into dissemination and variation of the health care-associated pathogen *Acinetobacter baumannii* from genomic analysis. *MBio* 5, e00963–e00913. doi: 10.1128/mBio.00963-13

Wu, X., Chavez, J. D., Schweppe, D. K., Zheng, C., Weisbrod, C. R., Eng, J. K., et al. (2016). *In vivo* protein interaction network analysis reveals porin-localized antibiotic inactivation in *Acinetobacter baumannii* strain AB5075. *Nat. Commun.* 7:13414. doi: 10.1038/ncomms 13414

Yamamoto, M., Nagao, M., Matsumura, Y., Matsushima, A., Ito, Y., Takakura, S., et al. (2011). Interspecies dissemination of a novel class 1 integron carrying $bla_{\text{IMP-19}}$ among *Acinetobacter* species in Japan. *J. Antimicrob. Chemother.* 66, 2480–2483. doi: 10.1093/jac/dkr336

Yang, H., Wang, M., Yu, J., and Wei, H. (2015). Antibacterial activity of a novel peptide-modified lysin against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Front. Microbiol.* 6:1471. doi: 10.3389/fmicb.2015.01471

Yang, Y. S., Lee, Y., Tseng, K. C., Huang, W. C., Chuang, M. F., Kuo, S. C., et al. (2016). *In vivo* and *in vitro* efficacy of minocycline-based combination therapy for minocycline-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 60, 4047–4054. doi: 10.1128/AAC.02994-15

Yokoyama, Y., Matsumoto, K., Ikawa, K., Watanabe, E., Morikawa, N., and Takeda, Y. (2015). Population pharmacokinetic-pharmacodynamic target attainment analysis of sulbactam in patients with impaired renal function: dosing considerations for *Acinetobacter baumannii* infections. *J. Infect. Chemother.* 21, 284–289. doi: 10.1016/j.jiac.2014.12.005

Yum, J. H., Yi, K., Lee, H., Yong, D., Lee, K., Kim, J. M., et al. (2002). Molecular characterization of metallo-β-lactamase-producing *Acinetobacter baumannii* and Acinetobacter genomospecies 3 from Korea: identification of two new integrons carrying the $bla_{\text{VIM-2}}$ gene cassettes. *J. Antimicrob. Chemother*. 49, 837–840. doi: 10.1093/jac/dkf043

Yu, Y. S., Zhou, H., Yang, Q., Chen, Y. G., and Li, L. J. (2007). Widespread occurrence of aminoglycoside resistance due to ArmA methylase in imipenem-resistant *Acinetobacter baumannii* isolates in China. *J. Antimicrob. Chemother.* 60, 454–455. doi: 10.1093/jac/dkm208

Zavascki, A. P., Goldani, L. Z., Li, J., and Nation, R. L. (2007). Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J. Antimicrob. Chemother.* 60, 1206–1215. doi: 10.1093/jac/dkm357

Zhang, X., Yang, T., Cao, J., Sun, J., Dai, W., and Zhang, L. (2016). Mucosal immunization with purified OmpA elicited protective immunity against infections caused by multidrug-resistant *Acinetobacter baumannii*. *Microb. Pathog.* 96, 20–25. doi: 10.1016/j.micpath.2016.04.019

Zhu, J., Wang, C., Wu, J., Jiang, R., Mi, Z., and Huang, Z. (2009). A novel aminoglycoside-modifying enzyme gene *aac(6′)-Ib* in a pandrug-resistant *Acinetobacter baumannii* strain. *J. Hosp. Infect.* 73, 184–185. doi: 10.1016/j.jhin.2009.05.012

Zimbler, D. L., Park, T. M., Arivett, B. A., Penwell, W. F., Greer, S. M., Woodruff, T. M., et al. (2012). Stress response and virulence functions of the *Acinetobacter baumannii* NfuA Fe-S scaffold protein. *J. Bacteriol.* 194, 2884–2893. doi: 10.1128/JB.00213-12

Zoued, A., Brunet, Y. R., Durand, E., Aschtgen, M. S., Logger, L., Douzi, B., et al. (2014). Architecture and assembly of the Type VI secretion system. *Biochim. Biophys. Acta* 1843, 1664–1673. doi: 10.1016/j.bbamcr.2014.03.018

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 03 April 2017
doi: 10.3389/fcimb.2017.00108

Contribution of the *A. baumannii* A1S_0114 Gene to the Interaction with Eukaryotic Cells and Virulence

Soraya Rumbo-Feal[1†], Astrid Pérez[1,2,3†], Theresa A. Ramelot[4†], Laura Álvarez-Fraga[1], Juan A. Vallejo[1], Alejandro Beceiro[1], Emily J. Ohneck[3], Brock A. Arivett[3], María Merino[1], Steven E. Fiester[3], Michael A. Kennedy[4], Luis A. Actis[3], Germán Bou[1]* and Margarita Poza[1]**

[1] Departamento de Microbiología, Instituto de Investigación Biomédica, Complejo Hospitalario Universitario (CHUAC), Universidad de A Coruña (UDC), A Coruña, Spain, [2] Departamento de Microbiología y Parasitología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain, [3] Department of Microbiology, Miami University, Oxford, OH, USA, [4] Department of Chemistry and Biochemistry, Miami University, Oxford, OH, USA

OPEN ACCESS

Edited by:
Ghassan M. Matar,
American University of Beirut, Lebanon

Reviewed by:
Sheryl S. Justice,
Ohio State University at Columbus, USA
J. Christopher Fenno,
University of Michigan, USA

***Correspondence:**
Luis A. Actis
actisla@miamioh.edu
Germán Bou
german.bou.arevalo@sergas.es
Margarita Poza
margarita.poza.dominguez@sergas.es

[†] These authors have contributed equally to this work.

Received: 24 November 2016
Accepted: 17 March 2017
Published: 03 April 2017

Citation:
Rumbo-Feal S, Pérez A, Ramelot TA, Álvarez-Fraga L, Vallejo JA, Beceiro A, Ohneck EJ, Arivett BA, Merino M, Fiester SE, Kennedy MA, Actis LA, Bou G and Poza M (2017) Contribution of the A. baumannii A1S_0114 Gene to the Interaction with Eukaryotic Cells and Virulence. Front. Cell. Infect. Microbiol. 7:108. doi: 10.3389/fcimb.2017.00108

Genetic and functional studies showed that some components of the *Acinetobacter baumannii* ATCC 17978 A1S_0112-A1S_0119 gene cluster are critical for biofilm biogenesis and surface motility. Recently, our group has shown that the A1S_0114 gene was involved in biofilm formation, a process related with pathogenesis. Confirming our previous results, microscopy images revealed that the ATCC 17978 Δ0114 derivative lacking this gene was unable to form a mature biofilm structure. Therefore, other bacterial phenotypes were analyzed to determine the role of this gene in the pathogenicity of *A. baumannii* ATCC 17978. The interaction of the ATCC 17978 parental strain and the Δ0114 mutant with A549 human alveolar epithelial cells was quantified revealing that the A1S_0114 gene was necessary for proper attachment to A549 cells. This dependency correlates with the negative effect of the A1S_0114 deletion on the expression of genes coding for surface proteins and pili-assembly systems, which are known to play a role in adhesion. Three different experimental animal models, including vertebrate and invertebrate hosts, confirmed the role of the A1S_0114 gene in virulence. All of the experimental infection assays indicated that the virulence of the ATCC 17978 was significantly reduced when this gene was inactivated. Finally, we discovered that the A1S_0114 gene was involved in the production of a small lipopeptide-like compound herein referred to as acinetin 505 (Ac-505). Ac-505 was isolated from ATCC 17978 spent media and its chemical structure was interpreted by mass spectrometry. Overall, our observations provide novel information on the role of the A1S_0114 gene in *A. baumannii*'s pathobiology and lay the foundation for future work to determine the mechanisms by which Ac-505, or possibly an Ac-505 precursor, could execute critical functions as a secondary metabolite.

Keywords: ***Acinetobacter baumannii*, biofilm, attachment, virulence, electron microscopy, secondary metabolite**

INTRODUCTION

Traditionally, *Acinetobacter baumannii* has been considered a low-virulence pathogen since its pathogenicity is influenced by the clinical condition of the patients it colonizes and infects. However, this epithet is often ignored by physicians due to its frequent and increasing occurrence as a multi-drug resistant (MDR) nosocomial pathogen around the world (Perez et al., 2008). In specific environments, such as intensive care and burn units where there is a remarkable selective antibiotic pressure, *A. baumannii* colonizes new niches because of its noteworthy ability to adapt to stressful conditions by modulating the expression of several virulence factors (Beceiro et al., 2013). Despite the importance of this microorganism, only a few virulence factors have been described to date (McConnell et al., 2013). This situation now can be addressed because of the development of inexpensive and convenient high-throughput sequencing methods, which have led to the description of numerous *A. baumannii* genomes and have facilitated the comparative analysis of entire genomes (Merino et al., 2014; Álvarez-Fraga et al., 2015; Ou et al., 2015). Furthermore, improved strategies for functional analysis of bacterial genes and the use of relevant animal models have provided novel insights into the virulence traits of this pathogen, which could lead to potential targets for the treatment of human infections (McConnell et al., 2013).

The inhibition of bacterial functions involved in quorum sensing, adhesion, colonization, iron acquisition and/or resistance to host defenses are possible strategies that could be used to fight bacterial infections, particularly those caused by MDR pathogens (Escaich, 2010). In the case of *A. baumannii*, one such strategy includes targeting capsular polysaccharides that have been identified as a virulence factors; capsule-deficient strains showed lower pathogenicity in a rat model (Russo et al., 2010). The acinetobactin-mediated iron acquisition system and related iron-mediated metabolic functions also play a role in *A. baumannii* ATCC 19606^T virulence as assessed using *ex vivo* and *in vivo* infection models (Gaddy et al., 2012; Zimbler et al., 2012, 2013). The ability of this pathogen to attach to different types of surfaces is also essential for its spread within the hospital environment and among patients as well as to colonize host tissues and medical devices. The outer membrane protein A (OmpA) and the biofilm-associated protein (Bap) are critical in host-pathogen interactions as well as in the interaction of bacteria with abiotic and biotic surfaces including human epithelial cells and neonatal keratinocytes (Choi et al., 2005, 2008; Iacono et al., 2008; Loehfelm et al., 2008; Kim et al., 2009). The *A. baumannii* ATCC 19606^T Type I pili assembled by the CsuA/BABCDE usher-chaperone assembly system were the first cellular appendages shown to be crucial for adherence and biofilm formation on abiotic surfaces under different experimental conditions (Tomaras et al., 2003). Our group previously identified the gene A1S_1507, which is part of a second Type I pili cluster, the disruption of which caused a significant decrease in biofilm formation by *A. baumannii* ATCC 17978 (Rumbo-Feal et al., 2013).

Comparative transcriptional studies of *A. baumannii* ATCC 17978 planktonic and sessile cells showed that expression of the A1S_0114 gene had the highest fold-change in biofilm-associated cells as compared to planktonic cells (Rumbo-Feal et al., 2013). Accordingly, deletion of this predicted gene led to a substantial decrease in biofilm formation (Rumbo-Feal et al., 2013). Random transposon mutagenesis of *A. nosocomialis* M2 resulted in the isolation of the M2-2 and M2-11 derivative mutants, which displayed a significant reduction in surface motility and harbored insertions in the *A. baumannii* ATCC 17978 A1S_0113 and A1S_0115 orthologs (Clemmer et al., 2011). Further RNA-Seq analysis and A1S_0112-*lacZ* fusion assays showed that the expression of the A1S_0112-A1S_0118 genes in *A. nosocomialis* M2 is transcriptionally activated by an AbaI-dependent quorum-sensing pathway (Clemmer et al., 2011). More recently, random insertion mutagenesis of the *A. baumannii* ATCC 17978hm, a hyper-motile derivative that harbors an IS insertion within the *hns*-like gene (Eijkelkamp et al., 2013; Giles et al., 2015), resulted in the isolation of the A1S_0112::Tn and A1S_0115::Tn mutants (Giles et al., 2015). Both mutants displayed no surface motility and a significant reduction in pellicle formation with an increased biofilm formation phenotype, observations suggesting a further link between motility and biofilm/pellicle formation (Giles et al., 2015). Based on all these observations, it was proposed that the A1S_0112-A1S_0118/0119 genes constitute a seven- or eight-gene operon, which is predicted to be involved in the biosynthesis of an uncharacterized secondary metabolite, such as a non-ribosomally synthesized lipopeptide (Clemmer et al., 2011; Eijkelkamp et al., 2013, 2014; Giles et al., 2015). Interestingly, this operon has been identified in many of the available genomic sequences of *A. baumannii*, including MDR strains (Adams et al., 2008; Iacono et al., 2008; Zhu et al., 2013), with the exception of the nonpathogenic *A. baumannii* SDF strain (Vallenet et al., 2008).

In this report we have established that the *A. baumannii* ATCC 17978 (referred to as 17978 in the rest of this work) A1S_0112-A1S_0119 gene cluster is indeed a polycistronic operon that includes eight genes predicted to code for proteins with functions involved in the production of bacterial secondary metabolites. Furthermore, a 17978 A1S_0114 isogenic deletion derivative (Δ0114) showed a significant reduction in cell adherence and virulence, as confirmed using three animal models. Mass spectrometry analysis of spent culture supernatants showed that the deletion of the A1S_0114 gene is associated with the absence of acinetin 505 (Ac-505), a 505-Da lipopeptide in which a hydroxylated-C_{15} acyl moiety is linked to both a Gly and a Cys-Gly containing moiety and has a non-standard peptide linkage. These observations indicate that the A1S_0114 gene could play a critical role in the pathobiology of *A. baumannii*, knowledge that could aid in the design of alternative therapeutic tools needed for the treatment of infections caused by emerging MDR isolates.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A. baumannii ATCC 17978 and *Escherichia coli* strains listed in **Table 1** were routinely grown or maintained in Luria-Bertani (LB) broth with 2% agar added for plates. All strains were grown at 37°C with shaking (180 rpm) and stored at -80°C

TABLE 1 | Bacterial strains and plasmids used in this work.

Strain or plasmid	Relevant characteristic(s)	Sources or references
STRAINS		
A. baumannii		
ATCC 17978	Clinical isolate	ATCC
Δ0114	ATCC 17978 A1S_0114 deletion derivative	This study
Δ0114.C	17978 Δ0114 harboring pWH1266-Km-0114; Km^r	This study
Δ0114.E	17978 Δ0114 harboring pWH1266-Km; Km^r, Tc^r	This study
E. coli		
TG1	Used for DNA recombinant methods	Lucigen
OP50	Used for maintenance of *C. elegans*; Ura^-, Str^r	CGC
PLASMIDS		
pCR-Blunt II-TOPO	Cloning vector; Km^r, Zeo^r	Invitrogen
pWH1266	*A. baumannii* shuttle vector; Ap^r, Tc^r	Stiernagle, 2006
pWH1266-Km	*A. baumannii* shuttle vector; Km^r, Tc^r	This study
pWH1266-Km-0114	pWH1266-Km harboring A1S_0114; Km^r	This study
pMo130	Suicide vector for construction of *A. baumannii* isogenic derivative; Km^r, SacB, XylE	Hamad et al., 2009

Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tet^R, tetracycline resistance; Str^R, streptomycin resistance; Zeo^R, zeocin resistance.

in LB broth containing 10% glycerol. Swimming broth (SB) containing 10 g/L of tryptone and 5 g/L of NaCl was used for some phenotypic analyses and 0.3% of agarose were added for plates (Harding et al., 2013). When appropriate, cultures were supplemented with kanamycin (Km) at a final concentration of 50 μg/mL. Bacterial growth curves were determined in sextuplet using 96-well microtiter plates containing either LB or SB inoculated with 17978 or Δ0114 cells under the aforementioned culturing conditions over a 24-h time period (**Figure S2**). OD_{600} values of these cultures were recorded hourly.

Construction of Isogenic Deletion Derivatives

Plasmid pMo130 (**Table 1**), a suicide vector containing the genes *xylE*, *sacB,* and a Km resistance marker, was used as described by Hamad et al. (2009). Briefly, 900–1,000 bp upstream and downstream regions flanking the genes selected for deletion in 17978 were PCR-amplified and cloned into the pMo130 vector using primers listed in **Table S1**. The resulting plasmid (pMo130-0114, shown in **Table 1**) was transformed into 17978 cells by electroporation (Rumbo-Feal et al., 2013). Recombinant colonies representing the first crossover event were selected by resistance to Km and visual detection of XylE activity following the catechol-based method (Hamad et al., 2009). Bright yellow Km resistant colonies were then grown overnight in LB supplemented with 15% sucrose and then plated on LB agar without antibiotics. The second crossover event leading to gene deletion was then confirmed by PCR using primers listed in **Table S1**. The Δ0114 isogenic deletion derivative of 17978 was constructed by deleting a region encompassing the A1S_0114 gene (Hamad et al., 2009).

Complementation of the Δ0114 Deletion Derivative

A kanamycin resistance marker was PCR-amplified from the pCR-BluntII-TOPO plasmid (from Invitrogen) using the primers listed in **Table S1**. The resulting product was inserted in the *Pst*I site of the pWH1266 plasmid (Hunger et al., 1990), obtaining the pWH1266-Km plasmid. To complement the Δ0114 strain, the A1S_0114 wild type allele was PCR-amplified from 17978 genomic DNA using the primers listed in **Table S1**. The resulting product was cloned into the *Eco*RV and *Bam*HI restriction sites of the pWH1266-Km plasmid (**Table 1**). The parental A1S_0114 allele was cloned as a *Bam*HI-*Eco*RV amplicon into the pWH1266-Km under the control of the tetracycline resistance gene promoter using the primers listed in **Table S1**. The complementing plasmid pWH1266-Km-0114 (**Table 1**) was transformed into Δ0114 cells by electroporation (Rumbo-Feal et al., 2013). Transformants were selected on Km-containing plates and the presence of pWH1266-Km-0114 was confirmed by PCR using primers listed in **Table S1**. Δ0114 cells harboring empty pWH1266-Km were used as a negative control.

RNA Extraction and Transcript Quantification

Cultures of 17978 and its derivatives were grown for 48 h at 37°C in SB. RNA was extracted with the Maxwell 16 LEV simplyRNA Cells Kit (Promega). The total RNA samples were treated using the DNA-free DNA Removal Kit (Ambion). The integrity of the RNA samples was checked using an Agilent 2100 Bioanalyzer and qPCR. cDNA was obtained from RNA samples using the iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's recommendations. The level of expression of particular genes was tested by real time PCR (qRT-PCR) using cDNA as a template and the KAPA SYBR FAST qPCR kit (Kapa Biosystems) following the instructions of the manufacturer with the primers listed in **Table S1**. Three independent biological replicates were each tested in triplicate. The expression level was standardized relative to the transcription level of the housekeeping gene *recA* which was established as 1. PCR reactions lacking cDNA were used as negative controls. The statistical significance of the differences was determined using a Student's *t*-test.

To confirm the polycistronic nature of the A1S_0112-A1S_0119 gene cluster, cDNA, obtained from total RNA through reverse transcription, was used as a template in PCR reactions with Taq DNA Polymerase (New England Biolabs) using pairs of primers designed to anneal to the 3′-end of every gene and the 5′-end of the next one (**Figure 1** and **Table S1**). Genomic DNA and total RNA without reverse transcription were used as templates for positive and negative controls, respectively, and the amplicons

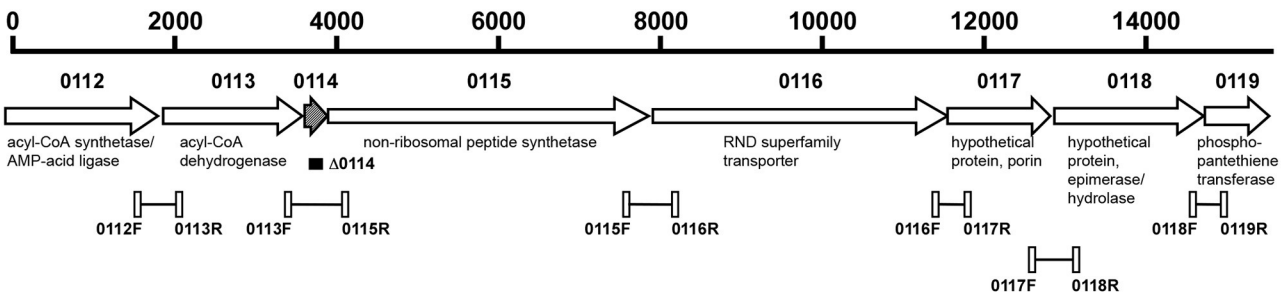


FIGURE 1 | Genetic organization of the A1S_0112-A1S_0119 operon of *A. baumannii* ATCC 17978. The horizontal arrows indicate the location and direction of transcription of the predicted genes. The numbers above the arrows represent the A1S_ genes located within this operon. The black rectangle indicates the region deleted within the A1S-0114 gene represented by the hatched arrow. The connected open rectangles and the number underneath of each of them identify the primers, listed in **Table S1**, and the regions amplified by PCR and RT-PCR assays using genomic DNA and DNA-free total RNA, respectively. Putative functions of the corresponding proteins are shown.

were detected by standard 1% agarose gel electrophoresis (Sambrook and Russell, 2001). These analyses were done in triplicate.

LC-MS Analysis of Bacterial Supernatants

The 17978 and Δ0114 strains were grown in static cultures and cell-free spent media was analyzed by liquid chromatography (LC)—mass spectrometry (MS) and LC-MS/MS with further internal source collision induced dissociation (ISCID) fragmentation. In brief, bacterial samples were cultured overnight in SB with shaking (200 rpm) at 37°C and used to inoculate SB at a 1:100 ratio. Then, 1-mL aliquots were dispensed into 12 × 75 mm polystyrene culture test tubes for incubation at 37°C for 48 h without shaking. Bacteria for isotopic labeling were cultured under the same conditions in unlabeled and U-^{15}N, 98% and U-^{15}N/^{13}C, 98% Bioexpress Cell Growth Media (Cambridge Isotope Laboratories, Inc.). Samples were vortexed, transferred to microcentrifuge tubes and centrifuged at 21,000 × g to remove cells. Aliquots were prepared for LC-MS by mixing 300 μL of spent media with 1:1 volume of 100% methanol (HPLC grade) containing 0.2% formic acid. LC-MS and LC-MS/MS (ISCID) data were collected on a micrOTOF Bruker Daltonics MS with Agilent Technologies 1200 Series LC and an analytical C_{18} column (100 × 2.1 mm, 2.6 mm particle size, Phenomenex Kinetex) operated at a column temperature of 35°C with a 0.2 mL/min flow rate. Solvent A was 100% water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. The elution method was a 30-min gradient from 100% solvent A to 90% solvent B in 25 min, followed by 5 min at 90% solvent B before returning to 100% solvent A for equilibration. Electrospray ionization (ESI)-MS was used in positive ion mode (*m/z* 50–2,000). Nitrogen was used as the drying gas with a temperature and nebulizing gas pressure of 190°C and 5.8 psi, respectively. The ESI capillary voltage of 4,500 V and Hexapole RF of 80 V_{pp} were used. ISCID energy of 80 eV (200 V) was applied for CID fragmentation. A sodium formate solution was employed as the external calibrant by direct infusion at the end of each run. LC-MS and LC-MS/MS (ISCID) data were analyzed using DataAnalysis 4.0 (Bruker Daltonics) software.

High-Resolution MS and MS^n Analysis of Ac-505

The 17978 strain was grown for 48 h at 37°C in SB in 2 L static cultures in flat-bottom flasks after a 1:100 dilution of innoculated shaken overnight culture. After centrifugation at 5,000 × g for 20 min to remove cells, 15 g of adsorptive XAD-7 resin was added to the culture supernatant and incubated at room temperature for 3 h. The resin was removed on glass filter paper in a Büchner funnel and then washed with 1 L deionized H_2O. The adsorbed material was then eluted with 300 ml of 100% methanol, dried on a rotary evaporator, and resuspended in 2 ml of methanol. Concentrated samples in methanol were kept at −80°C. One-ml injections were made on a BioCAD family vision Workstation equipped with a UV-detector, fraction collector, and a reversed-phase C_{18} preparative column (250 × 10.0 mm, 10 um particle size, Phenomenex Synergi Fusion-RP) operated at a 5 ml/min flow rate of the solvents A and B described above. The elution method was a gradient from 0 to 100% B in 10-column volumes, followed by an isocratic step with 100% solvent B for 5-column volumes. Eluted fractions of 1 ml were analyzed by analytical LC-MS on a Bruker micrOTOF (described above) and fractions containing purified Ac-505 were combined and dried using a stream of nitrogen.

High resolution LC-MS/MS in positive ion mode was performed at the Ohio State University (OSU) Mass Spectrometry and Proteomics Facility on a Bruker MaXis ESI Ionization Quadrupole Time-of-Flight Mass Spectrometer (ESI MaXis QTOF) with a Dionex U3000 RSLC system using a Waters Xbridge BEH C_{18} (3.5 μm, 1.0 × 100 mm) column and a gradient of H_2O and acetonitrile with 0.1% formic acid. Additionally, direct-infusion MS/MS in negative ion mode was performed on the ESI MaXis QTOF instrument. Ultra high resolution MS/MS was performed on the OSU ESI 15 tesla fourier transform ion cyclotron resonance (15T FT-ICR) instrument with electron-capture dissociation (ECD) of the

506.3 *m/z* peak in positive ion mode by direct infusion. MS^n fragmentation in positive ion mode was measured on the OSU Bruker amaZon, which has nominal mass resolution, using direct infusion of 5-μl samples of HPLC-purified Ac-505 in 50% methanol with 0.1% formic acid.

Bacterial Adhesion to A549 Human Alveolar Cells

A549 human alveolar epithelial cells were routinely maintained in 25-cm^2 tissue culture flasks in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum and 50 U/mL of penicillin and 50 μg/mL of streptomycin (DMEM) as described before (Gaddy et al., 2009). Confluent cultures were washed, trypsinized and transferred to 24-well plates to get a monolayer of 10^5 A549 cells *per* well. After 24 h of incubation under the same conditions, cells were washed twice with saline solution and once with modified Hank's balanced salt solution (mHBSS, same as HBSS but without glucose) following the protocol previously described (Gaddy et al., 2009). Then, the multiplicity of infection (MOI) of 10 was used; in each well 10^5 A549 cells were infected with 10^6 bacteria and incubated for 3 h in mHBSS at 37°C. To determine bacterial adhesion, the infected monolayers were washed three times with saline solution and then lysed in 500 μL of 0.5% sodium deoxycholate. Dilutions of the lysates were plated onto LB agar and incubated at 37°C for 24 h. Colony forming units were counted to determine the percent of bacteria that had attached to or invaded A549 cells as compared to the growth control. Six independent replicates were done. Student's *t*-test was performed to evaluate the statistical significance of the observed differences.

Electron Microscopy of Biofilms Formed on Plastic and Polarized A549 Cells

Sterile plastic coverslips were placed in sterile 50-mL conical tubes and then 5 ml of LB inoculated with each strain at a 1:100 dilution were added. Inoculated tubes were incubated for 48 h at 37°C without shaking as previously described (Gaddy et al., 2009). Coverslips were removed, washed, dehydrated in ethanol, processed with a critical point drier, and sputter coated as described previously (Tomaras et al., 2003). Biofilms formed above, at and below the liquid-air interface were viewed by scanning electron microscopy (SEM) using a Zeiss Supra Gemini Series 35 V scanning electron microscope as described previously (Tomaras et al., 2003).

A549 cells were polarized on the surface of Transwell 24-well permeable inserts as recently described (Álvarez-Fraga et al., 2016). Bacteria, previously grown in LB at 37°C for 24 h in a shaking incubator at 180 rpm, were washed and resuspended in Hank's Buffered Salt Solution (HBSS; Hyclone Laboratories, Inc.). An inoculum of 10^2 bacteria was applied to the apical surface of A549 cells by pipetting 1 μl of suspension onto the center of each membrane. The transwell plate was then incubated and maintained for 72 h at 37°C and 5% CO_2. After 72 h, the membranes were washed with HBSS to remove secretions and unattached bacteria. The membranes were then fixed for 24 h in 4% formaldehyde-HBSS at 4°C, prepared and viewed by SEM as previously described (Tomaras et al., 2003).

Caenorhabditis elegans Fertility Assay

Fertility assays were performed as previously described (Vallejo et al., 2015). Both the 17978 and Δ0114 strains were grown overnight in LB and then cultured at 37°C for 24 h in nematode growth medium (NG). The eggs of *C. elegans* N2 Bristol (a wild-type strain obtained from the CGC collection) were hatched in M9 medium (Vallejo et al., 2015), and worms in the first larval stage (L1) were arrested overnight at 20°C. Later, the L1 worms were added to the NG medium plates together with each bacterial strain selected for this study. One *C. elegans* worm in the last larval stage (L4) was placed on a peptone-glucose-sorbitol medium (PGS) plate individually seeded with each *A. baumannii* strain and incubated for 24 h at 25°C. The worms were transferred to new plates seeded with the same bacterial strain and the worm progeny was counted for 3 days to determine their viability. Six independent replicates were performed with each strain. Student's *t*-test was performed to evaluate the statistical significance of observed differences. Means of the differences between strains are reported.

Galleria mellonella Virulence Assay

A. baumannii cells previously grown for 24 h in LB broth were collected by centrifugation and resuspended in sterile phosphate-buffered saline (PBS). Appropriate bacterial inocula were estimated spectrophotometrically at OD_{600} and confirmed by plate counting using LB agar plates. To assess virulence, *G. mellonella* survival assays were performed by injecting in triplicate 10 randomly-selected healthy final instar *G. mellonella* larvae as previously described (Gaddy et al., 2012). The dose used for each infection consisted of a 5 μl-suspension containing 10^5 CFU (Gaddy et al., 2012). The control groups included larvae that either were not injected or were injected with the same volume of sterile PBS. The test groups included larvae infected with 17978 or Δ0114 bacteria. After injection, the larvae were incubated at 37°C in darkness, and death was assessed at 24-h intervals over 6 days. Caterpillars were considered dead, and were removed, if they displayed no response to probing. The resulting survival curves were plotted using the Kaplan-Meier method (Kaplan and Meier, 1958) and analyzed using the log-rank (Mantel-Cox) test. $P \leq 0.05$ were considered statistically significant (SAS Institute Inc.).

Murine Pneumonia Virulence Assay

A pneumonia model was used to evaluate the virulence of the 17978 and Δ0114 strains. BALB/c 9- to 11-week old female mice weighing 25–30 g were intratracheally infected with ∼5.5 × 10^7 CFUs/mouse of exponentially grown cells of the 17978 parental or the Δ0114 mutant strains into groups of 10 mice. Briefly, mice anesthetized with an oral suspension of sevoflurane (Abbott) were suspended by their incisors on a board in a semi-vertical position. Correct intratracheal inoculation was confirmed by using an endoscope on the oral cavity. The trachea was accessed using a blunt-tipped needle for the inoculation of a 40-μl bacterial suspension made in sterile saline solution

and 10% porcine mucin (wt/vol; Sigma) mixed at a 1:1 ratio. Dead mice in the first 4 h after inoculation were not included in the final analyses. Mice were euthanized with an overdose of thiopental sodium (Sandoz) 44 h after inoculation. Lungs were aseptically extracted, weighed, and homogenized in 1.5 ml of ice-cold saline solution in a Mixer Mill dismembrator (Retsch). Lung lysates were 10-fold serially diluted and samples were plated onto LB agar to measure organ bacterial loads. The results are shown as means of the $\log_{10}$ CFU *per* gram of lung with their standard deviations. Student's *t*-test was performed to evaluate the statistical significance of the observed differences. All mice were maintained in the specific pathogen-free facility at the Technology Training Center of the Hospital of A Coruña (CHUAC, Spain). All experiments were done with the approval of and in accordance with regulatory guidelines and standards set by the Animal Ethics Committee (CHUAC, Spain).

RESULTS

Characterization of the *A. baumannii* ATCC 17978 A1S_0112-0119 Operon

Previous transcriptional analysis of 17978 planktonic and sessile bacteria showed the differential expression of the A1S_0112-A1S_0118 genes, with A1S_0114 being expressed most highly by cells attached to an abiotic surface (Rumbo-Feal et al., 2013). *In silico* analysis of the chromosomal region harboring these genes indicates that it potentially codes for a polycistronic operon that includes the A1S_0119 coding region (**Figure 1**). This prediction is based on the observation that all putative genes are transcribed in the same direction and either overlap or are separated by intergenic regions ranging from 24 to 53 nucleotides according to reported genomic data (Smith et al., 2007) and our *in silico* analysis. The polycistronic nature of this operon was confirmed by RT-PCR analysis of total RNA using primers connecting the eight predicted genes. **Figure S1** shows the detection of the predicted amplicons when total RNA was reversed transcribed and PCR amplified using the primers shown in **Figure 1** and listed in **Table S1**, with their sizes matching those detected when total DNA was used as a template. In contrast, no amplicons were detected in any of the RNA samples that were PCR amplified without previous reverse transcription.

The analysis of 17978 genomic data showed that the 15,551-nt region harboring the A1S_0112-A1S_0119 predicted coding region is separated from an upstream *luxR* ortholog (A1S_0111) and a downstream tRNA-Gly (A1S_0120) gene by a 616-nt and a 95-nt non-coding region, respectively, with the latter gene being transcribed in the opposite direction.

Inactivation of A1S_0114 Affects Bacterial Interaction with Abiotic and Biotic Surfaces

In previous work we demonstrated the involvement of the A1S_0114 gene in the ability of 17978 cells to form biofilms on abiotic surfaces using crystal violet assays (Rumbo-Feal et al., 2013). This result is further supported in the present work by the analysis of biofilms formed on glass using SEM, which showed that 17978 cells attach to the abiotic surface and form multicellular structures associated with mature biofilms at the air-liquid interface (**Figures 2A,B**). In contrast, single or small cell clumps of the mutant derivative lacking the A1S_0114 gene (Δ0114) attached to the substratum without forming dense and three-dimensional structures (**Figures 2C,D**). It is important to note that the site-directed deletion of A1S_0114 did not affect the growth of the isogenic derivate when cultured either in Luria-Bertani (LB) or swimming broth (SB) without selective pressure (**Figure S2**).

The biological effect of the A1S_0114 deletion was also tested using A549 human alveolar epithelial cells as a model since they represent a host cell that could be targeted by *A. baumannii* during the pathogenesis of respiratory infections. To test the effect of the A1S_0114 deletion on bacterial adherence, submerged A549 confluent monolayers were co-incubated with 17978 or Δ0114 bacteria for 3 h and CFU counts were determined by plating serial dilutions of tissue culture cell lysates. This approach showed that the amount of Δ0114 bacteria recovered from A549 infected cells was 60% lower than that recovered from monolayers infected with 17978 bacteria ($P = 0.0198$; **Figure 3A**). Electroporation of Δ0114 cells with pWH1266-Km-0114, which harbors the A1S_0114 wild type allele expressed from the tetracycline resistance gene promoter, partially restored the adherence phenotype in the Δ0114.C derivative, a phenomenon that was not observed with Δ0114.E, a Δ0114 derivative transformed with the pWH1266-Km empty vector ($P = 0.0067$; **Figure 3A**).

The results obtained with submerged A549 monolayers were further confirmed by infecting A549 polarized cells with either the 17978 parental or the Δ0114 mutant strains. SEM analysis of A549 samples infected with 17978 bacteria revealed extensive damage to the surfactant layer that covers the polarized cells as well as to the epithelial cells themselves when compared with non-infected polarized cell samples (**Figure 3B**). Furthermore, micrograph 17978 shows the presence of numerous bacteria attached to the surface of cells or cell debris clearly seen after the surfactant layer was destroyed by bacterial action. Although the infection of A549 polarized samples by Δ0114 bacteria also resulted in destruction of the surfactant layer and cell damage, it appears that the deletion of A1S_0114 results in a readily detectable reduction of bacteria attached to the polarized samples (**Figure 3B**).

Role of A1S_0114 in the Expression of Genes Involved in Adherence and Biofilm Biogenesis

The observation that the deletion of A1S_0114 significantly affects bacterial adherence and biofilm biogenesis, prompted us to examine the differential expression of genes known or predicted to be involved in these *A. baumannii* cellular functions, including *ompA* and the *csuA/B* gene of the *csuA/BABCDE* pili assembly system (Tomaras et al., 2003; Gaddy et al., 2009; Cabral et al., 2011), and the genes A1S_0690, A1S_1510, and A1S_2091 that could be involved in bacteria-surface interactions (Rumbo-Feal et al., 2013; Eijkelkamp et al., 2014; Nait Chabane

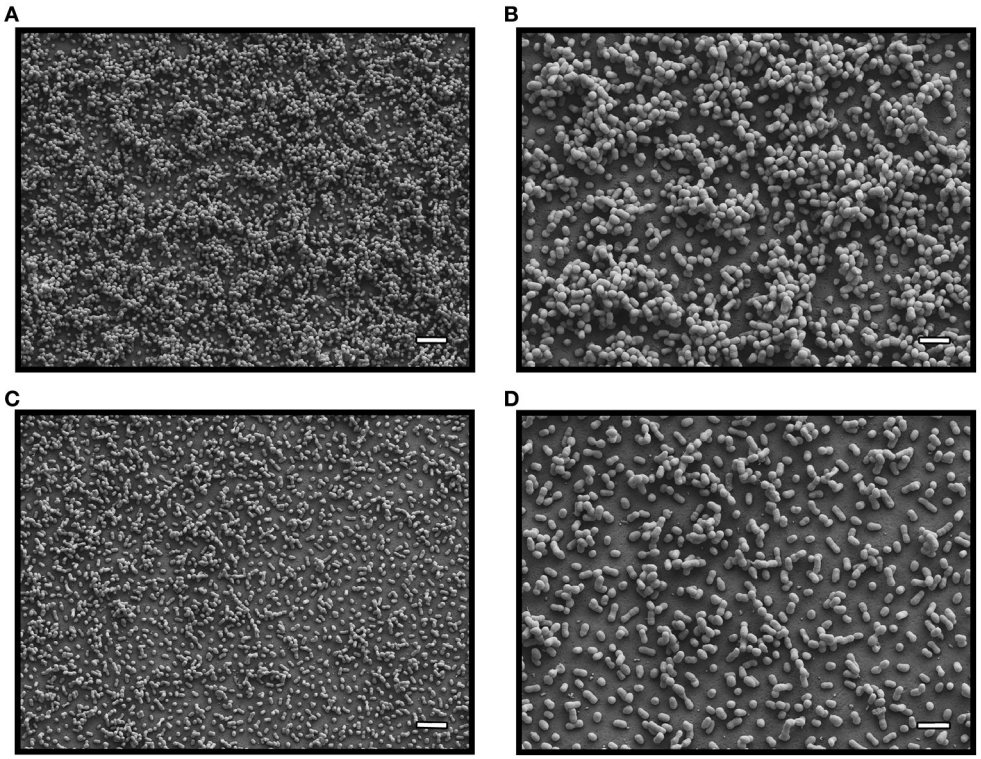


FIGURE 2 | SEM analysis of bacterial biofilms. Biofilms formed by 17978 (micrographs **A** and **B**) and Δ0114 (micrographs **C** and **D**) cells at the liquid-air interface. Micrographs A and C were recorded at a 5,000x magnification, while micrographs **B** and **D** were collected at a 10,000x magnification. White bars: 4 μm for **A**,**C**; 2 μm for **B,D**.

et al., 2014; Álvarez-Fraga et al., 2016). The comparative qRT-PCR analysis of total RNA isolated from 17978 and Δ0114 bacterial cells showed that the transcription of *csuA/B* is 5-fold increased in the Δ0114 mutant when compared with the parental wild-type strain (**Table 2**). In contrast, the transcriptional expression of the A1S_0690, A1S_1510, and A1S_2091 genes were significantly reduced, with A1S_2091 and A1S_1510 showing the highest (12-fold) and lowest (1.5-fold) changes, respectively. Deletion of A1S_0114 also caused a small (1.6-fold) but significant reduction in the transcription of *ompA*.

A1S_0114 Plays a Role in Virulence

The role of the A1S_0114 gene in the virulence of 17978 was assessed with a fertility assay using the *C. elegans* model, a survival assay using the caterpillar *G. mellonella* and a mouse pneumonia model, all of which have been previously used to examine the virulence of *A. baumannii*, particularly that of the 17978 strain (McConnell et al., 2013). *C. elegans* fertility assays showed that the total number of viable eggs was almost twice as high when worms were infected with Δ0114 when compared with 17978 (**Figure 4A**), with the difference between these two isogenic strains being statistically significant ($P < 0.0001$). Similarly, infection of *G. mellonella* larvae showed that *ca.* 50% of them died 5 days after being injected with 17978 (**Figure 4B**). This value was significantly different ($P = 0.024$) from that obtained with non-injected animals or animals injected with sterile PBS, which were used as negative controls. Although the infection of caterpillars with Δ0114 showed a killing rate that was significantly higher than the negative controls, the virulence of the mutant was significantly attenuated when compared with the parental strain ($P = 0.025$). Finally, the ability of the Δ0114 mutant to establish infection in an experimental murine model was evaluated. This model showed that lungs from mice infected with Δ0114 displayed a significantly lower bacterial burden than those from animals infected with 17978 ($P = 0.0165$) after 44 h of intratracheal infection (**Figure 4C**).

Identification and Characterization of the Secondary Metabolite Ac-505

The observation that the A1S_0114 gene plays a critical role in the pathobiology of *A. baumannii* prompted us to initiate a search for compounds that were produced by cells of the 17978 parental but not by cells of the Δ0114 mutant derivative. Liquid chromatography/mass spectrometry (LC-MS) analysis in positive ion mode of swimming broth (SB) static culture supernatants harvested at 48 h post inoculation showed the presence of a single peak with a retention time of ∼21 min and a molecular weight of 505.28 Da (*m/z* 506.29 $[M+H]^+$), which was consistently present in 17978 samples but not detected in samples from the

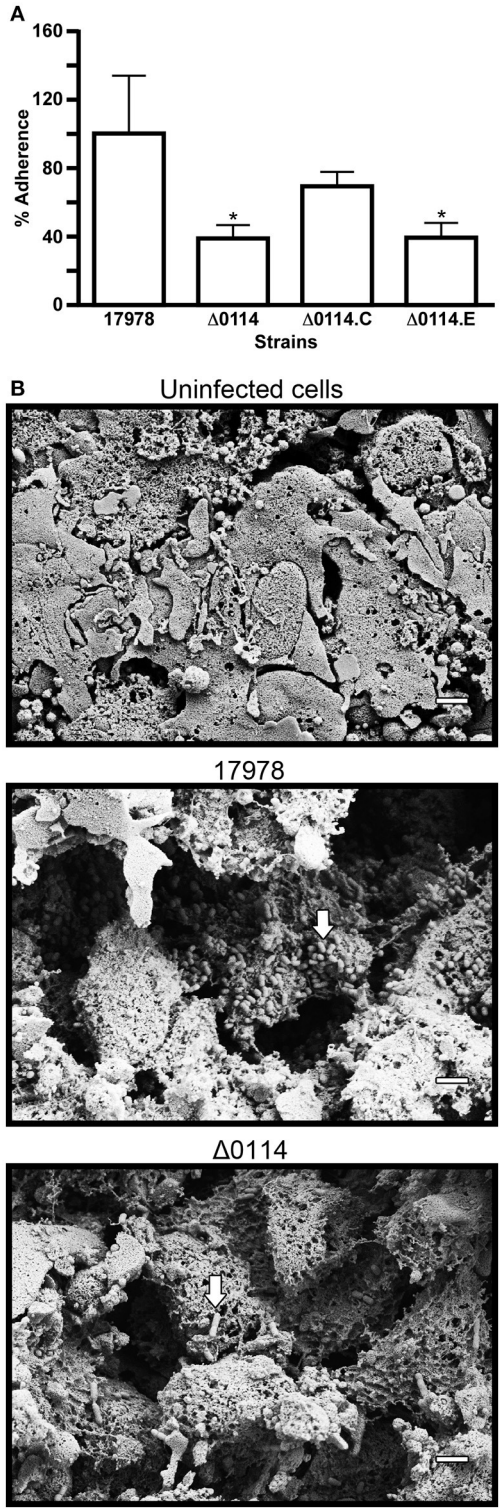


FIGURE 3 | Analysis of bacteria-human cell interactions. (A) Quantification of the adherence of 17978 and Δ0114 bacteria either not transformed or transformed with pWH1266-Km-0114, which harbors the A1S_0114 parental allele (Δ0114.C), or the empty shuttle vector pWH1266.Km (Δ0114.E) to polarized A549 cells. Bacterial adherence is *(Continued)*

FIGURE 3 | Continued
reported as the % of recovered bacteria compared to data collected with 17978, which was considered 100%. Data represent three independent replicates. Student's *t*-test was used to validate the experimental data, values are means and bars indicate standard deviation (*$P < 0.05$). **(B)** SEM analysis of A549 polarized cells either not infected (Uninfected cells) or infected with 17978 (17978) or with Δ0114 bacteria (Δ0114). All micrographs were taken at 10,000x magnification. White bars indicate the scale marks (2 μm). White arrows indicate bacteria attached to A549 cells or cell debris.

Δ0114 deletion derivative (**Figure 5A**). Based on this finding, we have named this compound acinetin 505 (Ac-505). Time course studies of SB cultures harvested every 24 h for 5 days (data not shown) showed a maximum amount of Ac-505 at 48 h post inoculation that leveled off. Further analysis of Ac-505 isolated and purified from 2 L of static SB culture supernatant showed that its molecular formula is $C_{23}H_{43}N_3O_7S$ based on ultra high-resolution 15T FT-ICR MS (*m/z* 506.28945 $[M+H]^+$, calculated 506.28945) as shown in **Figure 5B**. The number of predicted nitrogen and carbon atoms was further confirmed by growing 17978 cultures in unlabeled, and ^{15}N- and $^{15}N/^{13}C$-labeled media. LC-MS analysis of these culture supernatants confirmed the incorporation of 3 nitrogen and 23 carbon atoms in Ac-505 (*m/z* 506.3, 509.3, and 532.3 $[M+H]^+$, respectively). Additional high-resolution mass spectrometry (HRMS) analyses of FT-ICR ECD and MaXis QTOF MS/MS data are reported in **Tables S2**, **S3** and **Figures S3**, **S4C,D**, **S5**, **S6**. Nominal mass LC-MS^n fragmentation data from the Bruker amaZon were also used in the structural interpretation and two of the 40 spectra are shown in **Figures S4A,B**. Positive ion mode data were used to confirm neutral losses of water, two C-terminal glycines, cysteine, and cysteine-glycine as seen in **Figures S4A,C**. The MS/MS fragmentation data in both positive and negative ion mode correspond to data for other Cys-Gly conjugated compounds (Levsen et al., 2005). Specifically, the negative ion with 143.05 *m/z* is characteristic for detection of Cys-Gly conjugates (Dieckhaus et al., 2005). By analysis of the MS^n and FT-ICR fragmentation data, there is a neutral loss of water followed by loss of Cys-Gly and a second water leaving the remaining ion with the formula of $C_{18}H_{32}NO3^+$ (310.2 *m/z*) as seen in **Table S2** and **Figure S4C**. Further neutral loss of $C_2H_5NO_2$ from the 310.2 *m/z* ion matches a terminal glycine loss and the remaining 235.2 *m/z* ion has the formula $C_{16}H_{27}O^+$ (**Table S2**). The fragmentation patterns in the positive ion mode MS^n data for 310.2 and 235.2 *m/z* (shown in **Figure S4B**) had characteristic neutral losses of at least six 14 *m/z* corresponding to CH_2 losses, indicating a hydrocarbon chain of at least C_6. A neutral loss of $C_{14}H_{28}O$ from the 504.3 *m/z* parent ion was observed by MS/MS in negative ion mode as is shown in **Figure S3C**. Taken together, the MS data indicate that the structure of Ac-505 contains a Cys-Gly that is connected to the rest of the molecule via a sulfur linkage through the Cys side chain in a non-standard peptide linkage as shown in **Figure 5C**. The attachment of the Cys-Gly to the α-carbon of a Gly-containing moiety results in a so-called α-thio linkage. The Gly-containing moiety is connected to a hydroxylated acyl moiety of 15 carbons with a linkage that is neither *N*- or *O*-linked. The MS data supporting the

TABLE 2 | Expression level of genes *csuA/B*, A1S_2091, A1S_1510, A1S_0690, and *ompA* in *A. baumannii* ATCC 17978 and its mutant derivative 17978 Δ0114.

Gene[a]	ATCC 17978	17978 Δ0114	*P*-value
csuA/B	0.36 ± 0.14	1.81 ± 0.40	0.004
A1S_2091	2.64 ± 0.41	0.22 ± 0.04	0.0005
A1S_1510	0.86 ± 0.10	0.56 ± 0.04	0.0085
A1S_0690	8.48 ± 0.92	3.07 ± 0.38	0.0007
ompA	1.23 ± 0.14	0.76 ± 0.08	0.0071

[a] *The expression level of each gene was determined with respect to the expression level of recA, which was defined as 1.*

Values are means ± standard deviation. P-values indicate significant differences as determined by a Student's t-test.

structure shown in **Figure 5C** are detailed in **Tables S2**, **S3** and **Figures S4 –S6**.

DISCUSSION

A. baumannii has emerged as a pathogen with a remarkable ability to adapt and persist in response to a wide range of extracellular stimuli (Fiester and Actis, 2013). Such capacity relates to its genetic plasticity and the acquisition of genes coding for virulence-associated functions by horizontal gene transfer mechanisms. While some of these genes code for functions clearly related to bacterial pathogenicity (McConnell et al., 2013), there are other genes that code for potential virulence-associated traits whose mechanisms of action and biological roles are not fully understood. Among these genes, there is a gene cluster that was identified by our previous work as well as by other investigators using different *Acinetobacter* clinical isolates.

Study of the *A. baumannii* ATCC 17978, *A. baumannii* ATCC 17978hm and *A. nosocomialis* M2 strains resulted in the identification of the A1S_0112-A1S_0118 gene cluster while the analysis of the *A. baumannii* AB307-0294 isolate identified the ABBFA_003406-ABBFA_003399 cluster (Clemmer et al., 2011; Rumbo-Feal et al., 2013; Allen and Gulick, 2014; Giles et al., 2015). Importantly, these gene clusters code for cognate proteins that are highly related. Based on the nucleotide structure and the coding nature of the genes contained within this cluster of orthologous genes, it was hypothesized that it is either a 7- or 8-gene operon (Clemmer et al., 2011; Rumbo-Feal et al., 2013; Allen and Gulick, 2014; Giles et al., 2015). Transcriptional data presented in this report proved that this cluster is indeed a single 8-gene polycistronic transcriptional unit that encompasses the A1S_0112-A1S_0119 coding regions. This genetic structure explains the differential transcription reported for the A1S_0112-A1S_0118 genes in planktonic cells as compared to sessile cells (Rumbo-Feal et al., 2013). Our previous transcriptomic and mutagenesis studies also showed that site-directed deletion of A1S_0114, one of the highest transcribed genes in sessile cells when compared to exponential- and stationary-phase planktonic bacteria, caused a drastic reduction in biofilm biogenesis (Rumbo-Feal et al., 2013), a result that has been further confirmed in this work. The Δ0114 mutant not only forms less biofilms on plastic (**Figure 2**), but also shows

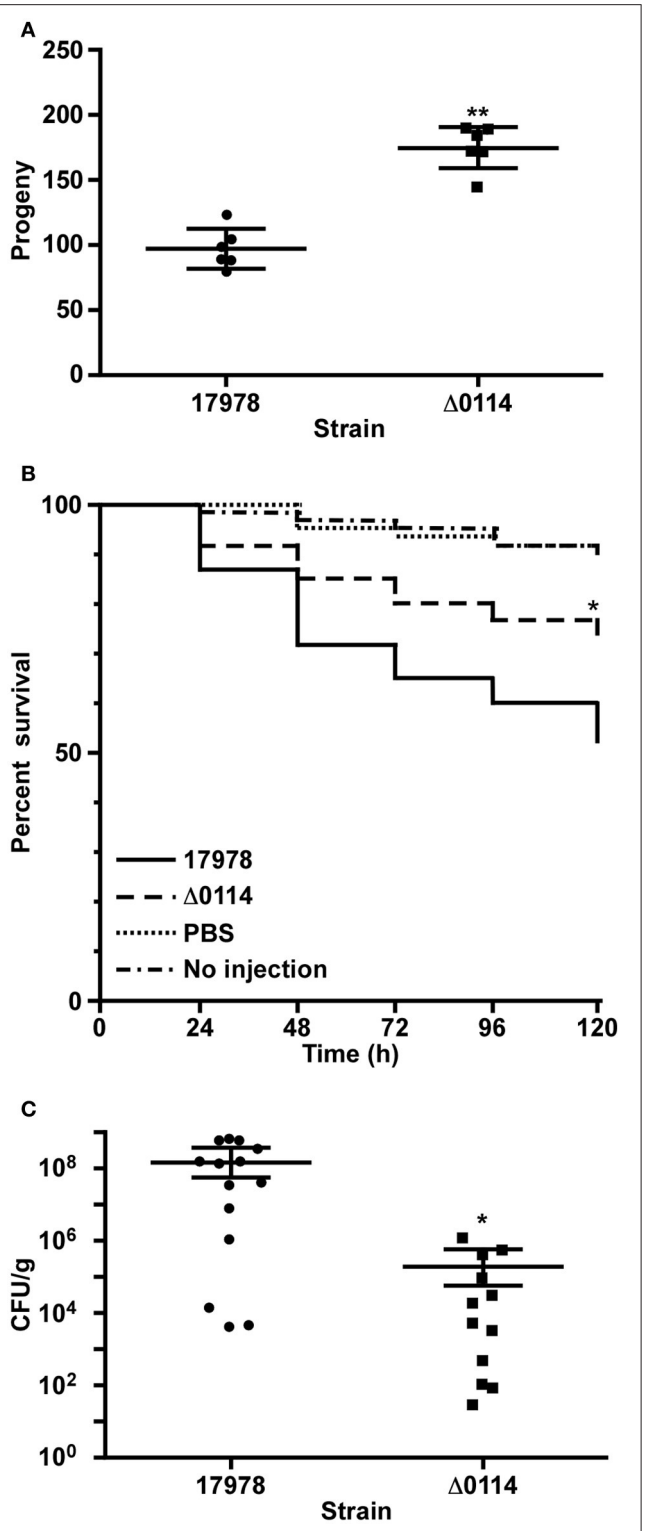


FIGURE 4 | Involvement of A1S_0114 in *A. baumannii* virulence. The virulence of the 17978 and Δ0114 strains was tested using *C. elegans* fertility and *G. mellonella* killing assays and a mouse pneumonia model. **(A)** *C. elegans* strain N2 was fed with 17978 or Δ0114 bacteria for 3 days counting progenies daily using six independent biological replicates.

(Continued)

FIGURE 4 | Continued
Each dot represents the worm progeny counted during 3 days. **(B)** *G. mellonella* caterpillars ($n = 60$ *per* group) were injected with 17978 or Δ0114 bacteria and death was determined daily for 5 days while being incubated 37°C in darkness. Caterpillars not injected or injected with the same volume of sterile PBS were used as negative controls. Log-rank (Mantel-Cox) tests were done to statistically validate experimental data. The asterisk in panel **(B)** indicates a $P < 0.05$ when the mutant and the wild type strains were compared. **(C)** Mice ($n = 10$ *per* group) were infected with $\sim 5.5 \times 10^7$ exponentially growing cells of the 17978 parental strain or the Δ0114 mutant *via* intratracheal intubation. The number of bacterial cells in lung homogenates was determined 24 h post infection. Student's *t*-test was used to validate experimental data shown in panels **(A,C)**. Values in panels **(A,C)** represent means and bars indicate the standard deviation (*$P < 0.05$; **$P < 0.001$).

less adherence when incubated with submerged or polarized human alveolar epithelial cells (**Figures 3A,B**). Interestingly, although the infection of polarized A549 cells with the Δ0114 mutant resulted in less biofilm formation on the surface of polarized cells, it did not result in an appreciable difference in the damage of the eukaryotic cells and the mucin layer that covers them when compared with samples incubated with the 17978 parent strain under the same experimental conditions (**Figure 3**). This observation suggests that A1S_0114 differentially affects distinct host-pathogen interactions that ultimately lead to the pathogenesis of respiratory infections caused by this pathogen.

If the phenotypes described above were to be pili mediated, as is the case of many bacteria, we would expect a correlation between the presence of an active A1S_0114 gene and the expression of genetic traits potentially involved in pili production and assembly. Accordingly, deletion of A1S_0114 significantly reduced the transcription of the A1S_0690, A1S_1510, and A1S_2091 genes (**Table 2**). A1S_0690 is a component of the A1S_0690-A1S_0695 operon, which codes for a predicted FilF-like Type III pili highly produced by pellicle cells (Marti et al., 2011; Nait Chabane et al., 2014); A1S_1510 is a component of the A1S_1507-A1S_1510 operon that codes for a predicted Type I pili, the production of which is controlled by iron and H-NS (Eijkelkamp et al., 2011a); and A1S_2091 is a component of the A1S_2088-A1S_2091 operon, which codes for an uncharacterized Type I pili also detected in pellicle cells (Nait Chabane et al., 2014; Álvarez-Fraga et al., 2016). Also, changes in biofilm biogenesis and adherence may reflect the fact that these responses could be mediated by the products of the *ompA* and *csuA/B* genes as described before (Tomaras et al., 2003; Gaddy et al., 2009). The expression of OmpA was reduced in the decreased biofilm former Δ0114 mutant, an observation that is in agreement with previous work (Gaddy et al., 2009). However, the biological relevance of the differential transcription of the *csuA/B* gene belonging to the *csuA/BABCDE* operon is not clear at the moment. It has been reported that this chaperone-usher pili assembly system may not be active in the 17978 strain due to a single base-pair insertion in *csuB* (Eijkelkamp et al., 2011b), although the CsuC and CsuD proteins have been detected as overproduced proteins in 17978 pellicle cells (Marti et al., 2011).

This report also provides the first experimental evidence that the A1S_0114 gene contributes to the virulence of *A. baumannii* when tested with three different experimental infection models already used to study *A. baumannii*'s virulence. It is apparent from our data that the marker-less deletion of A1S_0114, caused a significant reduction in virulence independently of the experimental infection model used to test this phenotype (**Figure 4**), without affecting the overall growth of this derivative in rich medium when compared to the 17978 parental strain (**Figure S2**).

It is interesting to note that a genome wide analysis using a random transposon mutant library containing 150,000 unique insertion derivatives, which included insertions in all components of the A1S_0112-A1S_0119 operon, showed that, in contrast to our observations, mutations within this operon did not affect the persistence of 17978 in the lungs of infected animals (Wang et al., 2014). Collection of samples at different times after infection (24 vs. 44 h) and the use of different mice strains (BALB/c vs. C57BL/6) could explain the disagreement between our data, collected using mutants generated by a site-directed approach, and that recorded with random transposon insertion derivatives. Similarly, different infection routes (pneumonia vs. bloodstream infection) and mice strains (BALB/c vs. leukopenic CBA/J) used to identified 17978 genes required for bacterial survival in the bloodstream of infected mice could explain the observation that mutants with random insertions in all A1S_0112-A1S_0119 genes did not meet the threshold to be considered critical for bacterial survival and no insertion within A1S_0114 was reported (Subashchandrabose et al., 2016). Genetic differences between the 17978 and AB5075 *A. baumannii* strains that resulted in apparent virulence differences using the *G. mellonella* as a host (Gebhardt et al., 2015) could explain the observation that none of the A1S_0112-A1S_0119 genes were identified as virulence genes when tested using this experimental infection model, which proved the critical role iron acquisition plays in *A. baumannii*'s virulence (Gaddy et al., 2012).

The putative functions for proteins encoded by this quorum-sensing regulated operon have been previously described (Clemmer et al., 2011) and include the acyl carrier protein (ACP) A1S_0114 and the predicted four-domain nonribosomal protein synthetase (NRPS) protein A1S_0115 (Allen and Gulick, 2014; Drake et al., 2016). The crystal structures of the *A. baumannii* AB307-0294 A1S_0115 orthologs, which shares 97.6% identity with the cognate 17978 gene product showed that glycine and AMP ligands were bound to the adenylation domain of this protein (PDB ID 4ZXI; Drake et al., 2016). Furthermore, glycine had the largest substrate specificity of the 20 proteinogenic amino acids tested, suggesting it could be the natural substrate incorporated into the biosynthetic product (Drake et al., 2016). A1S_0112 encodes an acyl-CoA synthase/AMP-acid ligase, which resembles fatty acid ACP ligases that activate and transfer fatty acids to ACPs *via* acyl AMP intermediates. A1S_0114, which encodes a free-standing ACP that is likely to contribute a tethered intermediate to the NRPS system involved in the biosynthesis of a secondary metabolite (Allen and Gulick, 2014), is the likely target for modification by A1S_0112, possibly yielding an acyl-ACP intermediate. A1S_0113, which encodes an acyl-CoA dehydrogenase (DH), is predicted to modify the intermediate product carried by the ACP or peptidyl carrier

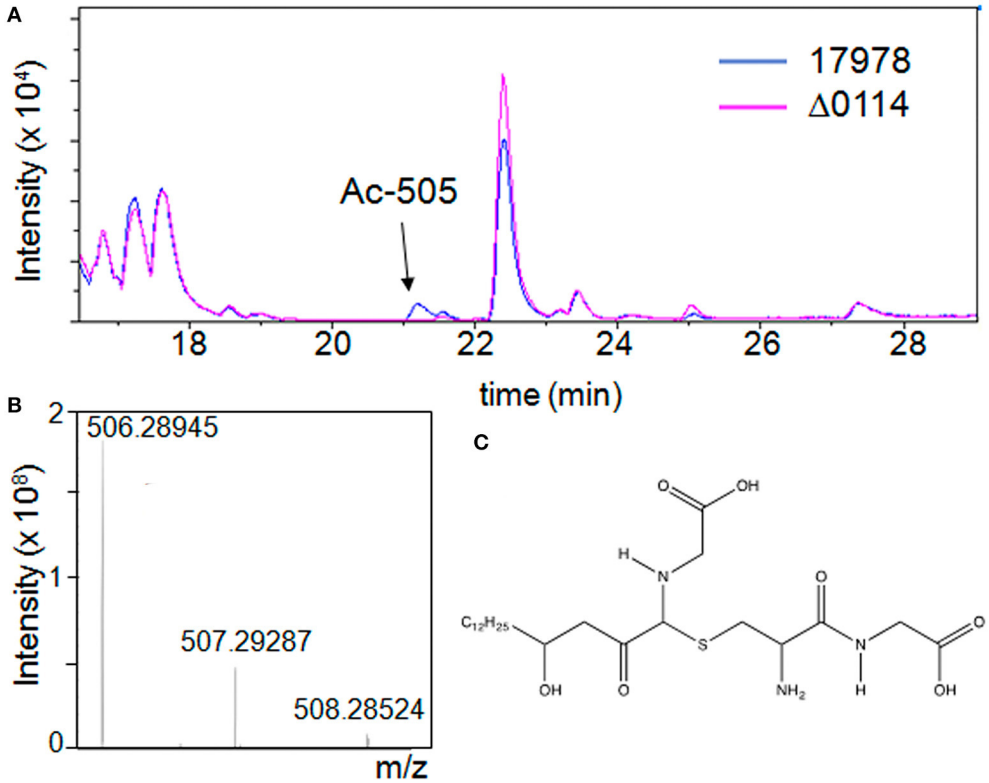


FIGURE 5 | Detection of Ac-505 in bacterial culture supernatants. (A) LC-MS chromatogram of culture supernatants from cells of the parental strain (17978) in blue and the A1S_0114 deletion derivative (Δ0114) in pink grown in static SB. The arrow indicates a compound (peak Ac-505) not present in the mutant profile. **(B)** Ultra high-resolution mass spectrum of HPLC purified Ac-505 by ESI FT-ICR showing the isotopic distribution of the $[M+H]^+$ ion. **(C)** Proposed structure of Ac-505.

protein (PCP) domain of A1S_0115. The thioesterase domain (TE) of A1S_0115 is most likely responsible for the release of acyl/peptide chains from their covalent attachment to the ACP/PCP domains. A1S_0117 and A1S_0118 encode 424- and 621-amino acid proteins, respectively, that were annotated as hypothetical proteins of unknown function (Smith et al., 2007). A more detailed *in silico* analysis showed that A1S_0117 could be related to porins while A1S_0118 harbors two domains that resemble proteins in the epimerase/dehydratase and α/β hydrolase family, respectively. However, since proteins in these families have diverse functions, the function of A1S_0118 still could not be predicted. The A1S_0119 gene, the last component of this operon, encodes a predicted 254-amino acid phosphopantetheine transferase that is expected to perform this enzymatic conversion, which is the first step in making the ACP active and therefore the likely first step in NRPS biosynthesis by this operon. Finally, the A1S_0116 gene encodes a protein that belongs to the superfamily of resistance-nodulation-cell division (RND) transporters. The function of these pumps may include efflux of signaling molecules, as is the case for the *Pseudomonas* quinolone signal called PQS (Lamarche and Deziel, 2011), and thus A1S_0116 is potentially involved in the secretion of the secondary metabolite coded for by the A1S_0112-A1S_0119 polycistronic operon. All these predictions are in accordance with our experimental observation that the expression of A1S_0114 gene not only is associated with the virulence of 17978, but also required for the production of the Ac-505 secondary metabolite.

Our mass spectrometric analyses of a purified compound present in 17978 culture supernatants but absent from those obtained from the Δ0114 mutant showed that Ac-505 resembles a three-amino acid lipopeptide, but with non-standard linkages between the amino acids as well as to the hydrocarbon moiety. Typically NRPS-produced secondary metabolites have hydroxylated or non-hydroxylated acyl moieties attached by amide or ester (*N*- or *O*-linked) linkages, which was not observed with Ac-505. We propose that the acyl-chain moiety or the neighboring Gly-containing component of Ac-505 could have been modified by other NRPS enzymes. Since it is possible for adenylation domains to be used iteratively to add sequential amino acids, it is conceivable that two or more Gly residues were incorporated via the A1S_0115 adenylation domain into this part of Ac-505. The Ac-505 second and third amino acids are Cys and Gly residues linked via a standard peptide linkage although they are connected to the Gly-containing moiety through the sulfur group of the Cys side chain forming a thioether bridge. Since we saw no evidence for unsaturation of the acyl chain, it is possible that the A1S_0113 DH acts in *cis* to modify the growing product on the glycine-containing moiety bound to the PCP of A1S_0115 rather than acting on an acyl-moiety of an acyl-ACP. The precursor to the α-thio bond could be an epoxide or a cyclic

compound, like a lactone. There are other NRPS-synthesized compounds that contain the so-called sactibiotic α-thio linkages such as the bacteriocins. These antimicrobial peptides contain a linkage between a cysteine thiol and the α-carbon of another amino acid residue. In the case of Ac-505, it is not clear how the α-thiol bond would be formed.

It is of note that the Ac-505 Cys-Gly moiety is potentially derived from glutathione, a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that is found in high concentrations intracellularly in *A. baumannii* and is linked with increased resistance to antibiotics (Kwon et al., 2013). Glutathione is known to deactivate xenobiotics *via* conjugation in order to make them less toxic. Glutathione conjugation most commonly occurs via a nucleophilic attack by the glutathione cysteinyl thiol on an electrophilic carbon such as a lactone or epoxide (Wang and Ballatori, 1998). The γ-glutamyl residue can then be cleaved by a γ-glutamyl transpeptidase localized outside the plasma membrane, leaving the conjugated Cys-Gly, as occurs in the case of the glutathione-modified microcystin (Schmidt et al., 2014), a cyanobacteria toxin and intra- and extra-cellular signaling molecule (Makower et al., 2015).

The Ac-505 that we detected both inside and outside 17978 cells, may be the result of the glutathione-mediated de-activation of a secondary metabolite. This potential precursor to Ac-505 would contain the hydrocarbon-chain and modified glycine-moiety and should have an electrophilic carbon, but the exact structure is unknown. Modification of this precursor by glutathione could explain our observation that no active fraction of the spent media would restore the wild type phenotype to the Δ0114 mutant, even with highly purified Ac-505 (data not shown). A similar outcome was obtained during analysis of the *A. nosocomialis* M2 strain and the isogenic derivatives M2-2 and M2-11, which harbor transposon insertions within the A1S_0113 and A1S_0115 orthologs, respectively, and display reduced surface motility (Clemmer et al., 2011). It is also possible that a different precursor to Ac-505 with a conjugated tri-peptide glutathione (prior to cleavage of the γ-glutamyl moiety), could be the active compound. We saw no evidence in the LC-MS analysis of spent media for the presence of a glutathione-conjugated derivative and were not able to identify any Ac-505 lipophilic precursors.

In summary, our work provides novel evidence that the A1S_0114 gene positively affects *A. baumannii* biofilm biogenesis, adherence and virulence responses. The molecular and cellular mechanism by which these responses are achieved and whether Ac-505 is the active effector responsible for these responses are issues that will remain unknown until this small secondary metabolite and its biosynthetic pathway are fully understood. Such knowledge would provide novel insights into the pathobiology of *A. baumannii* and potentially facilitate the development of alternative tools needed for the treatment of infections caused by MDR isolates.

AUTHOR CONTRIBUTIONS

SR: obtained the isogenic derivative and complemented strain and performed the qRT-PCR and attachment assays. AP: performed the scanning microscope assays and analyzed data of all work. TR: performed the LC-MS and high resolution MS and MS^n analysis. LÁ: performed the attachment assays. JV: performed the *C. elegans* and the mice virulence assays. AB: performed the mice virulence assays. EO: performed the scanning microscope experiments. BA: performed the *G. mellonella* virulence assay. MM: performed the attachment assays. SF: performed the complemented mutant strain analysis. MK: analyzed and supervised the work done in the Department of Chemistry and Biochemistry, Miami University, USA. LA: analyzed and supervised the work done in Department of Microbiology, Miami University, USA, interpreted all data and wrote the manuscript. GB: analyzed and supervised the work done in Spain. MP: coordinated and analyzed all the experiments of the work, interpreted data and wrote the manuscript.

FUNDING

This work has been funded by Projects PI15/00860 to GB, CP13/00226 to AB, PI11/01034 to MP and P14/000059 to MP and AB, all integrated in the National Plan for Scientific Research, Development and Technological Innovation 2013-2016 and funded by the ISCIII—General Subdirection of Assessment and Promotion of the Research—European Regional Development Fund (FEDER) "A way of making Europe." Miami University Research Funds from the departments of Microbiology and Chemistry and Biochemistry as well as funds from the College of Arts and Science Dean's office supported this work. We also want to thank the Spanish Network for Research in Infectious Diseases (REIPI RD12/0015/0014 to GB), co-financed by the European Development Regional Fund (EDRF) "A Way to Achieve Europe, Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad. JV was financially supported by the Sara Borrell Programme (ISCIII, Spain CD13/00373). SR was financially supported by the Agustí Pumarola Grant (Societat Catalana de Malalties Infeccioses i Microbiologia Clínica, SCMIMC) and Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). AP was financially supported by the Galician Plan for Research, Innovation and Growth (I2C Plan 2012-2016).

ACKNOWLEDGMENTS

We thank Arpad Somogyi and Cao Yu at the Ohio State University Mass Spectrometry and Proteomics Facility for invaluable discussion and MS data analysis. Finally, we thank M. I. Voskuil for providing pMo130.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00108/full#supplementary-material

Figure S1 | Confirmation of the polycistronic nature of the *A. baumannii* ATCC 17978 A1S_0112-A1S_0119 operon. Total DNA (lanes 2–7), cDNA (lanes 8–13) and total RNA (lanes 14–19) samples were used as templates in PCR

reactions using primers annealing to the A1S_0112-A1S 0113 (lanes 2, 8, and 14), A1S_0113-A1S_0115 (lanes 3, 9, and 15), A1S_0115-A1S_0116 (lanes 4, 10, and 16), A1S_0116-A1S_0117 (lanes 5, 11, and 17), A1S_0117-A1S_0118 (lanes 6, 12, and 18), and A1S_0118- A1S_0119 (lanes 7, 13, and 19) intergenic regions. Lanes 1 and 20 show the molecular weight standard Gene Ruler 1-Kb plus (Thermofisher Scientific). Molecular weight of each amplicon is indicated at the bottom of the figure.

Figure S2 | Growth of the 17978 parental and the Δ0114 isogenic deletion derivative strains. The OD_{600} values of each strain grown in LB or SB at 37°C for 24 h with shaking were determined hourly. Error bars represent the standard error (SE) of the mean.

Figure S3 | Ac-505 MS/MS spectra. (A) MS data collected on the FT-ICR with ECD fragmentation in positive ion mode. **(B)** MS/MS collected on a MaXis QTOF in positive ion mode (LC-MS) and **(C)** negative ion mode (direct injection).

Figure S4 | MS/MS—based fragmentation predictions (A,B) are two of the 40 total MS^n spectra from MS(3) to MS(6) recorded on the OSU Bruker amaZon in positive ion mode using direct injection of a HPLC purified Ac-505. MS(2) for 488.3 and MS(4) for 235.2 *m/z* fragment ions are shown in **(A,B)**, respectively. Neutral losses are shown in black. Panel **(C)** is a schematic diagram of MS/MS fragmentation of Ac-505 in positive ion mode and **(D)** is for negative ion mode.

Figure S5 | Predicted product ions of Ac-505 based on accurate mass and typical bond cleavage patterns under electrospray conditions in positive ion mode.

Figure S6 | Predicted product ions of Ac-505 based on accurate mass and typical bond cleavage patterns under electrospray conditions in negative ion mode.

Table S1 | Oligonucleotides used in the present study.

Table S2 | HRMS data of Ac-505. Measured m/z-values are reported for MS fragment ions measured on ESI MaXis QTOF (LC-MS2) and 15T FT-ICR ECD (direct infusion) instruments in positive ion mode, respectively. The molecular formulas of the product ions are based on accurate mass and isotopic pattern matching of the ions as well as typical bond cleavage patterns for electrospray ionization.

Table S3 | HRMS data of Ac-505 and MS2 fragments measured on a ESI MaXis QTOF (direct infusion) in negative ion mode.

REFERENCES

Adams, M. D., Goglin, K., Molyneaux, N., Hujer, K. M., Lavender, H., Jamison, J. J., et al. (2008). Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J. Bacteriol.* 190, 8053–8064. doi: 10.1128/jb.00834-08

Allen, C. L., and Gulick, A. M. (2014). Structural and bioinformatic characterization of an *Acinetobacter baumannii* type II carrier protein. *Acta Crystallogr. D Biol. Crystallogr.* 70(Pt 6), 1718–1725. doi: 10.1107/s1399004714008311

Álvarez-Fraga, L., López, M., Merino, M., Rumbo-Feal, S., Tomás, M., Bou, G., et al. (2015). Draft genome sequence of the biofilm-hyperproducing *Acinetobacter baumannii* clinical strain MAR002. *Genome Announc.* 3:e00824-15. doi: 10.1128/genomeA.00824-15

Álvarez-Fraga, L., Pérez, A., Rumbo-Feal, S., Merino, M., Vallejo, J. A., Ohneck, E. J., et al. (2016). Analysis of the role of the LH92_11085 gene of a biofilm hyper-producing *Acinetobacter baumannii* strain on biofilm formation and attachment to eukaryotic cells. *Virulence* 7, 443–455. doi: 10.1080/21505594.2016.1145335

Beceiro, A., Tomás, M., and Bou, G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin. Microbiol. Rev.* 26, 185–230. doi: 10.1128/cmr.00059-12

Cabral, M. P., Soares, N. C., Aranda, J., Parreira, J. R., Rumbo, C., Poza, M., et al. (2011). Proteomic and functional analyses reveal a unique lifestyle for *Acinetobacter baumannii* biofilms and a key role for histidine metabolism. *J. Proteome Res.* 10, 3399–3417. doi: 10.1021/pr101299j

Choi, C. H., Hyun, S. H., Lee, J. Y., Lee, J. S., Lee, Y. S., Kim, S. A., et al. (2008). *Acinetobacter baumannii* outer membrane protein A targets the nucleus and induces cytotoxicity. *Cell. Microbiol.* 10, 309–319. doi: 10.1111/j.1462-5822.2007.01041.x

Choi, C. H., Lee, E. Y., Lee, Y. C., Park, T. I., Kim, H. J., Hyun, S. H., et al. (2005). Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cell. Microbiol.* 7, 1127–1138. doi: 10.1111/j.1462-5822.2005.00538.x

Clemmer, K. M., Bonomo, R. A., and Rather, P. N. (2011). Genetic analysis of surface motility in *Acinetobacter baumannii*. *Microbiology* 157(Pt 9), 2534–2544. doi: 10.1099/mic.0.049791-0

Dieckhaus, C. M., Fernandez-Metzler, C. L., King, R., Krolikowski, P. H., and Baillie, T. A. (2005). Negative ion tandem mass spectrometry for the detection of glutathione conjugates. *Chem. Res. Toxicol.* 18, 630–638. doi: 10.1021/tx049741u

Drake, E. J., Miller, B. R., Shi, C., Tarrasch, J. T., Sundlov, J. A., Allen, C. L., et al. (2016). Structures of two distinct conformations of holo-non-ribosomal peptide synthetases. *Nature* 529, 235–238. doi: 10.1038/nature16163

Eijkelkamp, B. A., Hassan, K. A., Paulsen, I. T., and Brown, M. H. (2011a). Investigation of the human pathogen *Acinetobacter baumannii* under iron limiting conditions. *BMC Genomics* 12:126. doi: 10.1186/1471-2164-12-126

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Elbourne, L. D., Paulsen, I. T., and Brown, M. H. (2013). H-NS plays a role in expression of Acinetobacter baumannii virulence features. *Infect. Immun.* 81, 2574–2583. doi: 10.1128/iai.00065-13

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Papadimitrious, M. S., Paulsen, I. T., and Brown, M. H. (2011b). Adherence and motility characteristics of clinical *Acinetobacter baumannii* isolates. *FEMS Microbiol. Lett.* 323, 44–51. doi: 10.1111/j.1574-6968.2011.02362.x

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Paulsen, I. T., and Brown, M. H. (2014). Comparative analysis of surface-exposed virulence factors of *Acinetobacter baumannii*. *BMC Genomics* 15:1020. doi: 10.1186/1471-2164-15-1020

Escaich, S. (2010). Novel agents to inhibit microbial virulence and pathogenicity. *Expert Opin. Ther. Pat.* 20, 1401–1418. doi: 10.1517/13543776.2010.511176

Fiester, S. E., and Actis, L. A. (2013). Stress responses in the opportunistic pathogen *Acinetobacter baumannii*. *Future Microbiol.* 8, 353–365. doi: 10.2217/fmb.12.150

Gaddy, J. A., Arivett, B. A., McConnell, M. J., López-Rojas, R., Pachón, J., and Actis, L. A. (2012). Role of acinetobactin-mediated iron acquisition functions in the interaction of Acinetobacter baumannii strain ATCC 19606T with human lung epithelial cells, *Galleria mellonella* caterpillars, and mice. *Infect. Immun.* 80, 1015–1024. doi: 10.1128/IAI.06279-11

Gaddy, J. A., Tomaras, A. P., and Actis, L. A. (2009). The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect. Immun.* 77, 3150–3160. doi: 10.1128/iai.00096-09

Gebhardt, M. J., Gallagher, L. A., Jacobson, R. K., Usacheva, E. A., Peterson, L. R., Zurawski, D. V., et al. (2015). Joint transcriptional control of virulence and resistance to antibiotic and environmental stress in *Acinetobacter baumannii*. *MBio* 6, e01660–e01615. doi: 10.1128/mBio.01660-15

Giles, S. K., Stroeher, U. H., Eijkelkamp, B. A., and Brown, M. H. (2015). Identification of genes essential for pellicle formation in *Acinetobacter baumannii*. *BMC Microbiol.* 15:116. doi: 10.1186/s12866-015-0440-6

Hamad, M. A., Zajdowicz, S. L., Holmes, R. K., and Voskuil, M. I. (2009). An allelic exchange system for compliant genetic manipulation of the select agents *Burkholderia pseudomallei* and *Burkholderia mallei*. *Gene* 430, 123–131. doi: 10.1016/j.gene.2008.10.011

Harding, C. M., Tracy, E. N., Carruthers, M. D., Rather, P. N., Actis, L. A., and Munson, R. S. Jr. (2013). *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio* 4:e00360-13. doi: 10.1128/mBio.00360-13

Hunger, M., Schmucker, R., Kishan, V., and Hillen, W. (1990). Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. *Gene* 87, 45–51. doi: 10.1016/0378-1119(90)90494-C

Iacono, M., Villa, L., Fortini, D., Bordoni, R., Imperi, F., Bonnal, R. J., et al. (2008). Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the European clone II group. *Antimicrob. Agents Chemother.* 52, 2616–2625. doi: 10.1128/AAC.01643-07

Kaplan, E. L., and Meier, P. (1958). Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* 53, 457–481. doi: 10.2307/2281868

Kim, S. W., Choi, C. H., Moon, D. C., Jin, J. S., Lee, J. H., Shin, J. H., et al. (2009). Serum resistance of *Acinetobacter baumannii* through the binding of factor H to outer membrane proteins. *FEMS Microbiol. Lett.* 301, 224–231. doi: 10.1111/j.1574-6968.2009.01820.x

Kwon, D. H., Hekmaty, S., and Seecoomar, G. (2013). Homeostasis of glutathione is associated with polyamine-mediated beta-lactam susceptibility in *Acinetobacter baumannii* ATCC 19606. *Antimicrob. Agents Chemother.* 57, 5457–5461. doi: 10.1128/aac.00692-13

Lamarche, M. G., and Deziel, E. (2011). MexEF-OprN efflux pump exports the *Pseudomonas quinolone* signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). *PLoS ONE* 6:e24310. doi: 10.1371/journal.pone.0024310

Levsen, K., Schiebel, H. M., Behnke, B., Dötzer, R., Dreher, W., Elend, M., et al. (2005). Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview. *J. Chromatogr. A* 1067, 55–72. doi: 10.1016/j.chroma.2004.08.165

Loehfelm, T. W., Luke, N. R., and Campagnari, A. A. (2008). Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. *J. Bacteriol.* 190, 1036–1044. doi: 10.1128/jb.01416-07

Makower, A. K., Schuurmans, J. M., Groth, D., Zilliges, Y., Matthijs, H. C., and Dittmann, E. (2015). Transcriptomics-aided dissection of the intracellular and extracellular roles of microcystin in *Microcystis aeruginosa* PCC 7806. *Appl. Environ. Microbiol.* 81, 544–554. doi: 10.1128/aem.02601-14

Marti, S., Nait Chabane, Y., Alexandre, S., Coquet, L., Vila, J., Jouenne, T., et al. (2011). Growth of *Acinetobacter baumannii* in pellicle enhanced the expression of potential virulence factors. *PLoS ONE* 6:e26030. doi: 10.1371/journal.pone.0026030

McConnell, M. J., Actis, L., and Pachón, J. (2013). Acinetobacter baumannii: human infections, factors contributing to pathogenesis and animal models. *FEMS Microbiol. Rev.* 37, 130–155. doi: 10.1111/j.1574-6976.2012.00344.x

Merino, M., Alvarez-Fraga, L., Gómez, M. J., Aransay, A. M., Lavín, J. L., Chaves, F., et al. (2014). Complete genome sequence of the multiresistant *Acinetobacter baumannii* strain AbH12O-A2, isolated during a large outbreak in Spain. *Genome Announc.* 2:e01182-14. doi: 10.1128/genomeA.01182-14

Nait Chabane, Y., Marti, S., Rihouey, C., Alexandre, S., Hardouin, J., Lesouhaitier, O., et al. (2014). Characterisation of pellicles formed by *Acinetobacter baumannii* at the air-liquid interface. *PLoS ONE* 9:e111660. doi: 10.1371/journal.pone.0111660

Ou, H. Y., Kuang, S. N., He, X., Molgora, B. M., Ewing, P. J., Deng, Z., et al. (2015). Complete genome sequence of hypervirulent and outbreak-associated *Acinetobacter baumannii* strain LAC-4: epidemiology, resistance genetic determinants and potential virulence factors. *Sci. Rep.* 5:8643. doi: 10.1038/srep08643

Perez, F., Endimiani, A., and Bonomo, R. A. (2008). Why are we afraid of *Acinetobacter baumannii*? *Expert Rev. Anti Infect. Ther.* 6, 269–271. doi: 10.1586/14787210.6.3.269

Rumbo-Feal, S., Gómez, M. J., Gayoso, C., Álvarez-Fraga, L., Cabral, M. P., Aransay, A. M., et al. (2013). Whole transcriptome analysis of *Acinetobacter baumannii* assessed by RNA-sequencing reveals different mRNA expression profiles in biofilm compared to planktonic cells. *PLoS ONE* 8:e72968. doi: 10.1371/journal.pone.0072968

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., MacDonald, U., et al. (2010). The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. *Infect. Immun.* 78, 3993–4000. doi: 10.1128/iai.00366-10

Sambrook, J., and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*. New York, NY: Cold Spring Harbor.

Schmidt, J. R., Wilhelm, S. W., and Boyer, G. L. (2014). The fate of microcystins in the environment and challenges for monitoring. *Toxins* 6, 3354–3387. doi: 10.3390/toxins6123354

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M., et al. (2007). New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev.* 21, 601–614. doi: 10.1101/gad.1510307

Stiernagle, T. (2006). Maintenance of *C. elegans*. *WormBook* 1–11. doi: 10.1895/wormbook.1.101.1

Subashchandrabose, S., Smith, S., DeOrnellas, V., Crepin, S., Kole, M., Zahdeh, C., et al. (2016). *Acinetobacter baumannii* genes required for bacterial survival during bloodstream infection. *mSphere* 1:e00013-15. doi: 10.1128/mSphere.00013-15

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., and Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology* 149(Pt 12), 3473–3484. doi: 10.1099/mic.0.26541-0

Vallejo, J. A., Beceiro, A., Rumbo-Feal, S., Rodríguez-Palero, M. J., Russo, T. A., and Bou, G. (2015). Optimisation of the *Caenorhabditis elegans* model for studying the pathogenesis of opportunistic *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents.* doi: 10.1016/j.ijantimicag.2015.05.021. [Epub ahead of print].

Vallenet, D., Nordmann, P., Barbe, V., Poirel, L., Mangenot, S., Bataille, E., et al. (2008). Comparative analysis of Acinetobacters: three genomes for three lifestyles. *PLoS ONE* 3:e1805. doi: 10.1371/journal.pone.0001805

Wang, N., Ozer, E. A., Mandel, M. J., and Hauser, A. R. (2014). Genome-wide identification of *Acinetobacter baumannii* genes necessary for persistence in the lung. *MBio* 5, e01163–e01114. doi: 10.1128/mBio.01163-14

Wang, W., and Ballatori, N. (1998). *Endogenous glutathione* conjugates: occurrence and biological functions. *Pharmacol. Rev.* 50, 335–356.

Zhu, L., Yan, Z., Zhang, Z., Zhou, Q., Zhou, J., Wakeland, E. K., et al. (2013). Complete genome analysis of three *Acinetobacter baumannii* clinical isolates in China for insight into the diversification of drug resistance elements. *PLoS ONE* 8:e66584. doi: 10.1371/journal.pone.0066584

Zimbler, D. L., Arivett, B. A., Beckett, A. C., Menke, S. M., and Actis, L. A. (2013). Functional features of TonB energy transduction systems of *Acinetobacter baumannii*. *Infect. Immun.* 81, 3382–3394. doi: 10.1128/iai.00540-13

Zimbler, D. L., Park, T. M., Arivett, B. A., Penwell, W. F., Greer, S. M., Woodruff, T. M., et al. (2012). Stress response and virulence functions of the *Acinetobacter baumannii* NfuA Fe-S scaffold protein. *J. Bacteriol.* 194, 2884–2893. doi: 10.1128/jb.00213-12

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 09 May 2017
doi: 10.3389/fcimb.2017.00143

Response to Bile Salts in Clinical Strains of *Acinetobacter baumannii* Lacking the AdeABC Efflux Pump: Virulence Associated with Quorum Sensing

Maria López[1,2†], *Lucia Blasco*[1†], *Eva Gato*[1,2], *Astrid Perez*[1,2], *Laura Fernández-Garcia*[1,2], *Luis Martínez-Martinez*[2,3,4], *Felipe Fernández-Cuenca*[2,5,6], *Jesús Rodríguez-Baño*[2,5,6], *Alvaro Pascual*[2,5,6], *German Bou*[1,2], *and Maria Tomás*[1,2*] *on behalf of the GEIH-GEMARA (SEIMC)*

[1] *Department of Microbiology, Complejo Hospitalario Universitario A Coruña-INIBIC, La Coruña, Spain,* [2] *Spanish Network for Research in Infectious Diseases (REIPI RD12/0015), Hospital Virgen Macarena, Seville, Spain,* [3] *Department of Clinical Microbiology, Hospital Universitario Marqués de Valdecilla-IFIMAV, Santander, Spain,* [4] *Departament of Molecular Biology, University of Cantabria, Santander, Spain,* [5] *Department of Microbiology and Infectious Diseases, Hospital Universitario Virgen Macarena, Seville, Spain,* [6] *Department of Medicine, Universidad de Seville, Seville, Spain*

OPEN ACCESS

Edited by:
Ghassan M. Matar, American University of Beirut, Lebanon

Reviewed by:
Ziad Daoud, University of Balamand, Lebanon
Tânia Aparecida Tardelli Gomes, Universidade Federal de São Paulo, Brazil

***Correspondence:**
María Tomás
ma.del.mar.tomas.carmona@sergas.es

[†] *These authors have contributed equally to this work*

Received: *16 November 2016*
Accepted: *06 April 2017*
Published: *09 May 2017*

Citation:
López M, Blasco L, Gato E, Perez A, Fernández-Garcia L, Martínez-Martinez L, Fernández-Cuenca F, Rodríguez-Baño J, Pascual A, Bou G, and Tomás M (2017) Response to Bile Salts in Clinical Strains of Acinetobacter baumannii Lacking the AdeABC Efflux Pump: Virulence Associated with Quorum Sensing. Front. Cell. Infect. Microbiol. 7:143. doi: 10.3389/fcimb.2017.00143

Introduction: *Acinetobacter baumannii* is an opportunistic nosocomial pathogen associated with multiple infections. This pathogen usually colonizes (first stage of microbial infection) host tissues that are in contact with the external environment. As one of the sites of entry in human hosts is the gastrointestinal tract, the pathogen must be capable of tolerating bile salts. However, studies analyzing the molecular characteristics involved in the response to bile salts in clinical strains of *A. baumannii* are scarce.

Material and Methods: Microbiological and transcriptional studies (arrays and RT-PCR) in the response to bile salts were carried out in isogenic (*A. baumanni* Δ*adeB* ATCC 17978 and *A. baumannii* Δ*adeL* ATCC 17978) and clinical strains from clone ST79/PFGE-HUI-1 which is characterized by lacking the AdeABC efflux pump and by overexpression the AdeFGH efflux pump.

Results and Discussion: In presence of bile salts, in addition to the glutamate/aspartate transporter were found overexpressed in *A. baumannii* Δ*adeB* ATCC 17978, the virulence factors (surface motility, biofilm, and Type VI Secretion System) which are associated with activation of the Quorum Sensing system. Overexpression of these factors was confirmed in clinical strains of clone ST79/PFGE-HUI-1.

Conclusions: This the first study about the adaptive response to bile salts investigating the molecular and microbiological characteristics in response to bile salts of an isogenic model of *A. baumannii* ATCC 17978 and clinical isolates of *A. baumannii* (clinical strains

of ST79/PFGE-HUI-1) lacking the main RND efflux pump (AdeABC). Clinical isolates of *A. baumannii* lacking the AdeABC efflux pump (clone ST79/PFGE-HUI-1) displayed a new clinical profile (increased invasiveness) possibly associated with the response to stress conditions (such as the presence of bile salts).

Keywords: *Acinetobacter baumannii*, bile salts, quorum sensing, type VI secretion

INTRODUCTION

Acinetobacter baumannii is an important pathogen that is known to be a major agent in healthcare-associated and nosocomial infections (Antunes et al., 2013). The pathogen is increasingly involved in hospital outbreaks of infection, particularly in Intensive Care Units (ICUs) (del Mar Tomas et al., 2005). In most of these outbreaks, various inanimate objects in the hospital environment have been identified as the principal source of infection (Sherertz and Sullivan, 1985; Cefai et al., 1990). However, the genus *Acinetobacter* is known to be a normal inhabitant of human skin. Some researchers have therefore postulated that, in the context of an outbreak of *A. baumannii* infection, humans may be transient skin carriers, thus facilitating cross-contamination and also representing a potential source of hospital spread of the infection (Mulin et al., 1995). The digestive tract of ICU patients is an important reservoir of multiresistant *A. baumannii* in hospital settings (Corbella et al., 1996). The types of surveillance samples most frequently analyzed in patients include sputum and tracheostomy exudate, wounds, armpit/groin, and rectal smears. In a Spanish study involving the detection of *A. baumannii* in different surveillance samples from ICU patients, the microorganism was identified in 75% of axillary-pharyngeal samples and in 77% of rectal swabs; the pathogen was identified in 90% of patients following analysis of a combination of axillary/rectal/pharyngeal specimens and in 96% of patients following analysis of a combination of pharyngeal-rectal samples (Rodríguez-Baño et al., 2004).

Several factors affect the ability of the microorganism to persist in high numbers in the gut, both as a commensal and as an opportunistic pathogen. One such factor is tolerance to bile salts (Pumbwe et al., 2007). Bile salts (i.e., salts of bile acids) are formed from secondary bile acids (bile acids conjugated to amino acids) which attach to a sodium or potassium ion to form a salt. Bile acids are retained in the gallbladder as bile salts and are secreted into the intestine (Malik, 2016). Two mechanisms of tolerance to bile salts have been identified in bacteria to date: RND efflux pumps (Lin et al., 2003; Pumbwe et al., 2008) and glutamate transporters (Krastel et al., 2010).

Three types of RND efflux pumps have been described in clinical strains of *A. baumannii*: AdeABC (expression of which is controlled by AdeRS); AdeIJK (in which the regulatory gene is *adeN*); and AdeFGH (in which *adeL* is the negative regulatory gene). The AdeFGH pump and in particular the AdeABC pump play a major role in acquired resistance (Coyne et al., 2010; He et al., 2015), whereas the AdeIJK pump is responsible for intrinsic resistance (Coyne et al., 2011). Moreover, overexpression of AdeABC and AdeFGH efflux pumps has been associated with increased biofilm production (He et al., 2015; Yoon et al., 2015; Richmond et al., 2016). However, neither these RND efflux pumps nor glutamate transporters have been associated with tolerance to bile salts in *A. baumannii* strains.

Finally, several studies have investigated how the virulence factors associated with the Quorum Sensing (QS) system can be regulated differently throughout the intestine by bile salts and, therefore, by the different commensal bacteria present (Zheng et al., 2010; Bachmann et al., 2015). The QS system enables bacterial populations to live and proliferate in an environment (sometimes hostile) with effective intercellular communication. However, this has not yet been investigated in strains of *A. baumannii*.

In this study, we carried out microbiological and transcriptional studies to investigate the response to bile salts in clinical strains of *A. baumannii* (clone ST79/PFGE-HUI-1) lacking the AdeABC efflux pump, as well as in isogenic mutant strains of *A. baumannii* ATCC 17978.

MATERIALS AND METHODS

Strains, Susceptibility Testing, and Growth with Bile Salts

Isogenic and Clinical Strains

A. baumannii ATCC 17978 was used as a reference strain. This strain was used to produce two stable mutants with the pMo130 plasmid; following the instructions of Hamad and colleagues (Hamad et al., 2009), *A. baumannii* Δ*adeB* ATCC 17978 and *A. baumannii* Δ*adeL* ATCC 17978 mutants were obtained. The mutants were confirmed by sequencing analysis and RT-PCR assays (Rumbo et al., 2013). The primers used are listed in **Table S1** (Supplementary Material).

The Ab421 GEIH-2010 strain and other 10 clinical strains of *A. baumannii,* all belonging to clone ST79/PFGE-HUI-1 and identified during the second multicenter Spanish study of this pathogen (GEIH-REIPI-2010-Ab project), were included in the present study. These isolates were characterized in a previous study (Rumbo et al., 2013). Species identification was confirmed by detection of the bla_{OXA51} gene, and the *adeA, adeB, adeC, adeR,* and *adeS* genes were not detected (Rumbo et al., 2013).

To confirm the absence of the AdeABC system and regulatory genes, we applied Next Generation Sequencing (NGS) to a representative clinical strain (Ab421 GEIH-2010) of clone ST79/PFGE-HUI-1. The genome of this strain was recently published in the Genome Announcements (Lopez et al., 2016). Moreover, the LysR-type regulator protein (AdeL) upstream of the AdeF protein (AdeFGH) contained a new mutation that introduced an amino acid substitution (Met7→Stop) (Lopez et al., 2016).

The antibiotic susceptibility profile (isogenic and Ab421 GEIH-2010 strain) was determined by microdilution, according to CLSI recommendations (CLSI, 2015). The MICs were determined in the presence of bile salts (cholic acid sodium salt 50% and deoxycholic acid sodium salt 50%, Sigma Aldrich, Germany) and Phe-Arg β-naphthylamide dihydrochloride (PAbetaN), a commonly assumed RND efflux pump inhibitor (Pannek et al., 2006). The bile salts were used at a concentration 0.5% as the physiological concentration in the human intestine ranges between 0.1 and 1.3% (Pumbwe et al., 2007).

Bacterial Growth in the Presence of Bile Salts

Clinical strains (Ab421, Ab427, Ab428, Ab435, and Ab436) of *A. baumannii* clone ST79/PFGE-HUI-1 (two biological replicates of each strain) were grown in LB at 37°C and 180 rpm. After incubation of the cultures overnight, the optical density (OD) was measured and adjusted to 0.02 OD_{600} in modified LB-LN (medium low nutrients comprising 2 g/L tryptone, 1 g/L yeast extract, and 5 g/L NaCl) supplemented with bile salts at 0.5%. Cultures were incubated at 37° (static conditions) and the growth was monitory at different times (3, 6, 9, 12, 24, 36, and 48 h) in a Zuzi 4250/20 spectrophotometer (Jin et al., 1998) until an OD_{600} 0.4 was reached. The *A. baumannii* ATCC 17978 strain was included as a control.

Surface Motility

Motility assays were performed in 6-well plates containing three types of Luria broth: (i) normal LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl); (ii) modified LB-LS (medium low salts which is constituted by 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) (47); and (iii) modified LB-LN plus 0.3% Eiken agar (López et al., 2017). These media were supplemented with 0.5 and 1% bile salts, except for the controls (no supplementation).

Strains *A. baumannii* ATCC 17978 (and mutants thereof) and Ab421 GEIH-2010 were inoculated in LB broth (Normal LB, Modified LB-LS, and Modified LB-LN) and incubated overnight at 37°C. An aliquot of 1 μl of the overnight culture was spotted in the center of each well and the plates were incubated at 37°C. Migration was measured after overnight incubation of the culture. The average diameter of the zone of surface motility was determined, and the isolates were classified as non-motile (NM, <5 mm), intermediately motile (IM, 5–20 mm), and highly motile (HM, >20 mm).

Biolfim Experiments

Scanning Electron Microscopy (SEM) Studies

Overnight cultures (two biological replicates of each strain) of *A. baumannii* were used to inoculate 5 mL of modified LB-LN in 50 ml conical tubes at a 1:100 dilution. The test medium was supplemented with 0.5% bile salts, and the control medium was not supplemented. Sterile polystyrene coverslips were placed in the inoculated 50 mL conical tubes, which were incubated for 48 h at 37°C without shaking, as previously described (Gaddy et al., 2009). Coverslips were washed, dehydrated in ethanol, processed with a critical point drier, and sputter-coated, as described above (Tomaras et al., 2003). Biofilms formed above, at and below the liquid-air interface were viewed in a Zeiss Supra Gemini Series 35 V scanning electron microscope, as previously described (Rey et al., 1989).

Quantitative Assays

Biofilm formation was quantified following the procedure described by Álvarez-Fraga et al. (2016). The strains were grown on Luria Broth for 18 h at 37°C and used to inoculate 5 mL of LB broth. Cultures were grown at 37°C with shaking. Overnight cultures were pelleted, washed and resuspended in 5 mL of modified LB-LN in presence and absence of 0.5% bile salts. A 1:100 dilution of each strain was incubated at 37°C for 48 h under static conditions. Growth of the culture was measured at OD_{600} to estimate total cell biomass. Biofilm formation was quantified by staining with crystal violet and solubilized with ethanol-acetone. The OD_{580}/OD_{600} ratio was used to normalize the amount of biofilm formed to the total cell content of each sample tested, to overcome variations due to differences in bacterial growth under several experimental conditions. Eight independent replicates were considered. A student's *t*-test was performed to evaluate the statistical significance of the observed differences between the strains considered.

Gene Expression

Gene expression studies were carried out by microarray and RT-PCR analysis. In both types of analysis, RNA was isolated using hot phenol extraction and subjected to DNase I treatment (Invitrogen). The RNA was then cleaned on an RNeasy column (Qiagen) following the manufacturer's mini cleanup protocol (Hamner et al., 2013) to obtain Dnase-treated RNA from late log-phase cultures in LB-LN (OD = 0.4–0.6) in the absence and presence of 0.5% bile salts in static conditions and tigecyline (0.5 mg/L). The RNA samples were quantified in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The quality and integrity of the samples were determined in an Agilent 2100 Bioanalyzer with RNA 6000 Nano reagents and RNA Nano Chips (Agilent Technologies), and only samples with an RNA integrity number (RIN) >8 were included. Analysis of controls without reverse transcriptase confirmed the absence of contaminating DNA in the samples.

The *A. baumannii* strain ATCC 17978 and mutants *A. baumannii* Δ*adeL* ATCC 17978 and *A. baumannii* Δ*adeB* ATCC 17978 were included in the microarrays (Bioarray Diagnostico Genetico, Alicante, Spain). The analysis was conducted using eArray (Agilent) (Aranda et al., 2013). Labeling was carried out by two-color microarray-based prokaryote analysis by Fair Play III labeling, version 1.3 (Agilent). Four independent RNA extractions per condition (biological replicates) were used in each experiment. Statistical analysis was carried out with the Bioconductor software package RankProd for the R computing environment. A gene was considered induced when the ratio of the treated to the untreated preparation was ≥1.5 and the *P*-value was < 0.05.

For RT-PCR assays, we used the Ab421 GEIH-2010 and other clinical isolates (Ab427, Ab428, Ab435, and Ab436) of ST79/PFGE-HUI-1 clone and *A. baumannii* Δ*adeL* ATCC 17978 strains. The following were analyzed by RT-PCR: (i) expression

of the *adeG* gene (AdeFGH efflux pump) in the strains cultured in the presence of bile salts (0.5%) and tigecycline (0.5 mg/L), and (ii) expression of genes determined by microarray analysis (A1S_1490, A1S_0115, A1S_1295, and A1S_1510). The studies were carried out with the Lightcycler 480 RNA MasterHydrolysis Probe (Roche, Germany). The UPL Taqman Probes (Universal Probe Library-Roche, Germany) and primers used are listed in **Table S1**.

The concentrations of the samples were adjusted to efficiencies of 90–110% (50 ng of RNA), and all experiments were performed in triplicate from three RNA extractions. For each strain, the expression of all genes was normalized relative to that of the *rpoB* gene. The normalized expression of each gene of interest was then calibrated relative to its expression by strains cultured in the absence of the bile salts or tigecycline in *A. baumannii* ATCC 17978, which were assigned a value of 1.0 (Mean Relative Expression, RE) (Aranda et al., 2013). Overexpression of the gene was defined by RE values of $\geq$ 1.5 (Tomas et al., 2010).

RESULTS

Antimicrobial Susceptibility in *A. baumannii* ATCC 17978 Isogenic Model and Ab421 GEIH-2010 (Table 1)

Resistance of the *A. baumannii* strains to several antimicrobials increased in the presence of 0.5% bile salts, although not significantly, which may indicate that expression of the AdeFGH efflux pump (RND type) is not modulated by bile salts.

The MICs of tobramycin, tigecycline, norfloxacin, ciprofloxacin, gentamicin, tetracycline, and netilmicin were lowered in *A. baumannii* strain Δ*adeL* ATCC 17978 in the presence of the RND-efflux pump inhibitor Phe-Arg β-naphthylamide dihydrochloride (PAbetaN). These results confirm the overexpression of the AdeFGH efflux pump in relation to antibiotic resistance. Moreover, the levels of expression of the *adeG* gene (AdeFGH) revealed by RT-PCR explained the results of the MIC assays. The relative expression (RE) of the *adeG* gene in *A. baumannii* Δ*adeL* ATCC 17978 strains cultured in the presence of 0.5% bile salts was 0.5 (i.e., it was not overexpressed relative to expression in the absence of bile salts). However, the level of expression of *adeG* was 2.53 times higher in the presence than in the absence of tigecycline (0.5 mg/L).

In clinical strain Ab421 GEIH-2010 (belonging to clone ST79/PFGE-HUI-1), the MICs of tobramycin, tigecycline, norfloxacin, ciprofloxacin, gentamicin, tetracycline, and netilmicin decreased (by 2–4 times) in the presence of PAbetaN. The RE was also 2.71 times higher in the presence than in the absence of tigecycline (0.5 mg/L) in this isolate.

Virulence Phenotype: Surface Motility and Biofilm (*A. baumannii* ATCC 17978 Isogenic Model and Ab421 GEIH-2010)

In relation to surface motility studies in Normal LB (**Figure 1**), the *A. baumannii* ATCC and *A. baumannii* ATCC 17978Δ*adeL* strains did not grow, while *A. baumannii* Δ*adeB* ATCC 17978 and Ab421 GEIH-2010 both grew in the presence of 0.5 and 1% bile salts. The surface motility of isogenic strains (*A. baumannii* ATCC 17978 and mutants) was also higher in the presence of bile salts (0.5%) in this modified medium-Low Salt (LB-LS). However, the greatest increase in the motility of strain Ab421 GEIH-2010 cultured in presence of bile salts (0.5%) was observed in modified LB-Low Nutrients (LB-LN). Therefore, this concentration of bile salts (0.5%) and the modified medium LB-LN were used for all further experiments in this study.

In the biofilm assays, the presence of bile salts (0.5% in LB-LN) increased the capacity of biofilm production in Scanning Electron Microscopy (SEM) studies (**Figure 2**) and quantitative assays (Student's *t*-test, $P < 0.05$) in all strains considered in this study (**Figure 3**). However, different phases of biofilm formation were observed in the biofilm matrix in Ab421 GEIH-2010 and microcolonies (slime layer) in *A. baumannii* Δ*adeB* ATCC 17978 cells (**Figure 2**: SEM analysis). Moreover, these isolates showed the highest capacity for biofilm production in quantitative assays (**Figure 3**).

Gene Expression in Relation to Bile Salts in the *A. baumannii* ATCC 17978 Isogenic Model: Microarray Analysis

Microarray analysis yielded the following results (GEO database GSE85264).

A) Comparison of *A. baumannii* ATCC 17978 Isogenic Strains Cultured in the Presence of 0.5% Bile Salts (LB-LN)

Gene expression in the presence of bile salts (0.5%) in isogenic *A. baumannii* strains is summarized in **Table 2**. In the first experiment comparing *A. baumannii* ATCC 17978Δ*adeL* and the *A. baumannii* wild type strain, only one gene was overexpressed (*lysR* regulator family) in the mutant strain. This may be related to tolerance to bile salts in this isolate, although this was not confirmed. However, comparison of *A. baumannii* ATCC 17978Δ*adeB* and the *A. baumannii* wild type strain showed that seven genes were overexpressed in mutant strains in relation to acid tolerance (glutamate/aspartate transporter), gene mobility (transposases) and surface motility/biofilm formation (csuA/B). Finally, 25 genes associated with acid tolerance (glutamate/aspartate transporters), quorum sensing (acyl-CoA dehydrogenase, acyl-CoA synthase/AMP-acid ligases II, amino acid adenylation, acyl carrier protein, and RND superfamily transporter), iron/sulfur metabolism (ring hydroxylating dioxygenase Rieske [2Fe-2S] and aromatic-ring-hydroxylating dioxylating dioxygenase ß subunit), gene mobility (transposases), T6SS/Type VI Secretion System (vipA, hcp-1, putative signal peptide, putative membrane, and vipB), and motility/biofilm formation (csuA/B and fimbrial protein) were revealed by comparing *A. baumannii* ATCC 17978Δ*adeB* and *A. baumannii* ATCC 17978Δ*adeL*.

B) Comparison of Each Isolate Cultured in the Absence and Presence of Bile Salts (LB-LN)

Use of arrays to investigate gene expression in *A. baumannii* ATCC and mutant strains (*A. baumannii* Δ*adeB* ATCC 17978 and *A. baumannii* Δ*adeL* ATCC 17978) cultured in the absence and presence of bile salts (0.5%) revealed similar

TABLE 1 | MICs of different antimicrobial agents against clinical and isogenic strains of *A. baumannii* in the presence or absence of bile salts (0.5%) or in the presence of Phe-Arg β-naphthylamide dihydrochloride (PAbetaN, 100 μg/mL).

Strain	Antimicrobial	MIC (mg/L)	MIC (mg/L-Bile Salts 0.5%)	MIC (mg/L-PAβN 100 μg/mL)
A. baumannii ATCC 17978	Tobramycin	0.5	0.5	0.5
	Sulfamethoxazole	4,864	4,864	4,864
	Tigecycline	1	2	2
	Norfloxacin	4	2	4
	Ciprofloxacin	0.5	0.25	0.25
	Gentamicin	1	1	1
	Tetracycline	4	1	1
	Netilmicin	1	1	1
A. baumannii ATCC 17978 Δ*adeB*	Tobramycin	0.25	1	0.5
	Sulfamethoxazole	4,864	4,864	4,864
	Tigecycline	1	1	1
	Norfloxacin	4	4	4
	Ciprofloxacin	0.5	0.5	0.125
	Gentamicin	0.5	2	0.5
	Tetracycline	4	4	1
	Netilmicin	1	1	0.5
A. baumannii ATCC 17978 Δ*adeL*	Tobramycin	1	1	0.25
	Sulfamethoxazole	4,864	4,864	4,864
	Tigecycline	1	0.5	0.25
	Norfloxacin	8	8	0.5
	Ciprofloxacin	0.5	2	0.25
	Gentamicin	1	4	0.5
	Tetracycline	4	4	1
	Netilmicin	2	1	0.5
Ab421 GEIH-2010	Tobramycin	64	64	8
	Sulfamethoxazole	9,728	9,728	4,864
	Tigecycline	16	16	4
	Norfloxacin	1,024	2,048	512
	Ciprofloxacin	512	512	512
	Gentamicin	512	512	128
	Tetracycline	16	32	8
	Netilmicin	256	256	8

gene expression in all three isolates. Genes involved in the following processes were expressed under stress conditions: (i) Ferric iron binding (ii) Oxidoreductase/Transferase (iii) Isomerase/Fumarylacetoacetate, (iv) Response to toxic substances, and (v) DNA metabolism (**Table 3**).

Both results (A and B) of the microarray analysis are consistent with findings of motility studies in modified LB-LN (**Figure 1**).

Growth Curves on Presence of Bile Salts (0.5% in LB-LN) in Clinical Strains of the ST79/PFGE-HUI-1 Clone

To analyze the results obtained with isogenic models and Ab421 GEIH-2010, we used five clinical strains of *A. baumannii* belonging to the clone ST79/PFGE-HUI-1 (including the Ab421 GEIH-2010).

Interestingly, in the first 12 h, the *A. baumannii* clinical strains from PFGE-HUI-1 clone showed a greater growth than the strain *A. baumannii* ATCC 17978. However, after this time, the clinical strains had a stagnation (from 12 to 24 h). After 24 h, *A. baumannii* clinical strains from PFGE-HUI-1 and the *A. baumannii* ATCC 17978 strain reached to an OD_{600} of 0.5 and 0.3 (the bacterial growth was inoculated on LB plates). This may indicate the development of biofilm formation in the clinical strains from PFGE-HUI-1 clone not being possible the study of growth by optical density (**Figure 4**).

Gene Expression in Relation to Bile Salts in Clinical Strains of ST79/PFGE-HUI-1 Clone as Revealed by RT-PCR

Gene overexpression detected by microarray analysis was confirmed by RT-PCR in clinical strains of clone

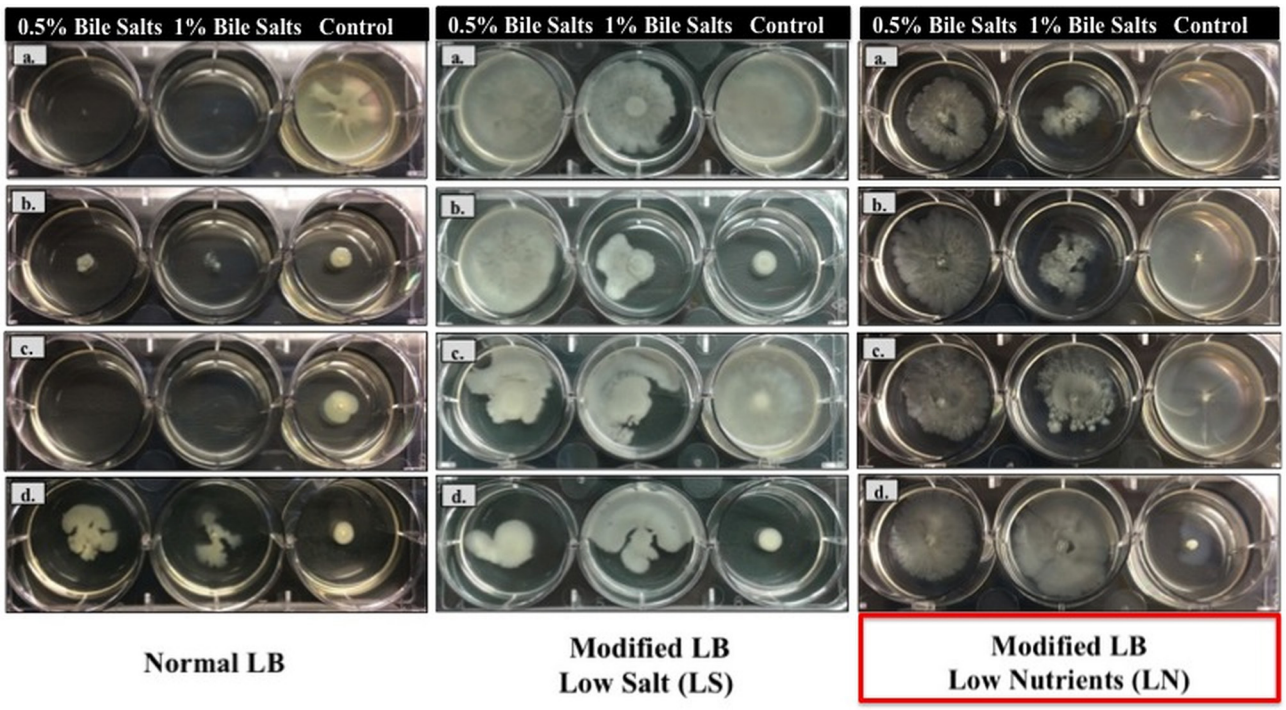


FIGURE 1 | Motility of *A. baumannii* strains in the presence of 0.5 and 1% of bile salts in three types of LB broth (Normal LB medium, Modified LB-LS and Modified LB-LN). (a) *A. baumannii* ATCC 17978; **(b)** *A. baumannii* Δ*adeB* ATCC 17978; **(c)** *A. baumannii* Δ*adeL* ATCC 17978; and **(d)** Ab421 GEIH-2010. The arrows on the left show the similarity in the behavior of both strains (*A. baumannii* Δ*adeB* ATCC 17978 and Ab421 GEIH-2010) in different medium and in presence of bile salts.

ST79/PFGE-HUI-1 (in addition to the Ab421 GEIH-2010). The levels of overexpression (RE) of A1S_1490 (Acid tolerance), A1S_0115 (Quorum Sensing), A1S_1295 (T6SS), and A1S_1510 (Surface motility/Biofilm formation) were statistically significantly higher in clinical isolates of the ST79/PFGE-HUI-1 clone cultured in the presence of bile salts (0.5%) than in the same clone cultured in LB-LN (**Table 4**). These results obtained with the clinical strains of the PFGE-HUI clone confirmed the overexpression of genes involved in the response to bile salts in the isogenic model of *A. baumannii* ATCC 17978. Moreover, all strains of this ST79/PFGE-HUI-1 clone (17 isolates) were considered to be the cause of infection in all six patients. Interestingly, five of the six patients had bacteraemia (primary or secondary).

DISCUSSION

The RND type of multidrug efflux pumps play several roles in bacterial pathogens: (i) provision of resistance to antimicrobial and antiseptic compounds, including those naturally present in mucosa; (ii) regulation of virulence factors via involvement in quorum-sensing regulation; (iii) detoxification of intracellular metabolites; and finally, (iv) mediation of cell homeostasis and intercellular signal trafficking (Beceiro et al., 2013).

In *A. baumannii*, the AdeABC RND-pump is the main efflux system involved in antimicrobial resistance. However, 25–30% of clinical strains of *A. baumannii* do not possess the AdeABC efflux pump (Chu et al., 2006; Lin et al., 2009).

In the GEIH-REIPI-2010 Ab Project (a multicentre study in which 45 hospitals participated), we studied clinical strain Ab421 GEIH-2010, which lacks the AdeABC efflux pump as well as regulatory genes and also overexpresses the AdeFGH system. This clinical strain belongs to clone ST79/PFGE-HUI-1 (seventeen isolates) (Rumbo et al., 2013). The mechanisms associated to the adaptive response to bile salts were analyzed in mutant strains of *A. baumannii* ATCC 17978 by considering an isogenic model (*A. baumannii* Δ*adeB* ATCC 17978 and *A. baumannii* Δ*adeL* ATCC 17978). Interestingly, the Ab421 GEIH-2010 strain displayed a higher capacity for surface motility and biofilm formation when cultured in the presence of bile salts than when cultured in the absence of these.

The mechanisms associated to the response to bile salts (essential for survival in the gastrointestinal tract) are not well-known in *A. baumannii*. In a study of the profile of protein overexpression in response to monovalent cations (200 mM of NaCl), Hood et al. observed glutamate/aspartate transport (Hood et al., 2010). Moreover, glutamate transport (glnQHMP operon) has also been implicated with acid tolerance in *Streptococcus mutans* (Krastel et al., 2010). Our results with *A. baumannii* Δ*adeB* ATCC 17978 (microarray analysis) and clinical strains of ST79/PFGE-HUI-1, including strain Ab421 GEIH-2010 (RT-PCR), revealed the involvement of this

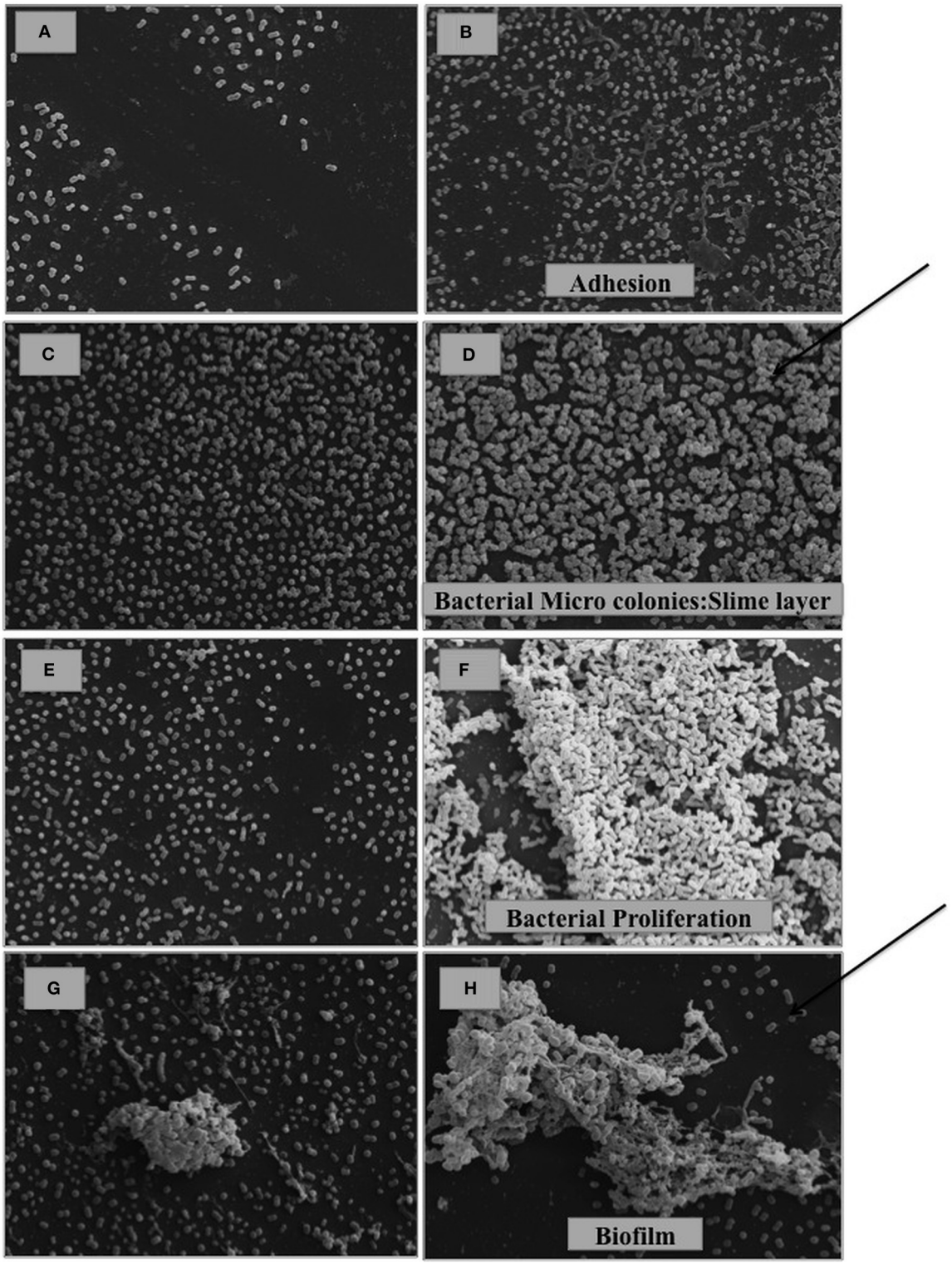


FIGURE 2 | SEM analysis of *A. baumannii* cells cultured in the absence (A,C,E,G) and presence of 0.5% bile salts **(B,D,F,H)**. **(A,B)** *A. baumannii* ATCC 17978; **(C,D)** *A. baumannii* Δ*adeB* ATCC 17978; **(E,F)** *A. baumannii* Δ*adeL* ATCC 17978; **(G,H)** Ab421 GEIH-2010. (Scale bars: 20 μm). It is observed in presence of bile salts, the state of adhesion in *A. baumannii* ATCC 17978 **(B)**, slime layer-micro colonies (previous state of biofilm formation) in *A. baumannii* Δ*ade*B ATCC 17978 **(D)**, proliferation in *A. baumannii* Δ*ade*L ATCC 17978 **(F)** and finally, biofilm formation in Ab421 GEIH-2010 **(H)**.The arrows indicate the most advanced stages of biofilm development.

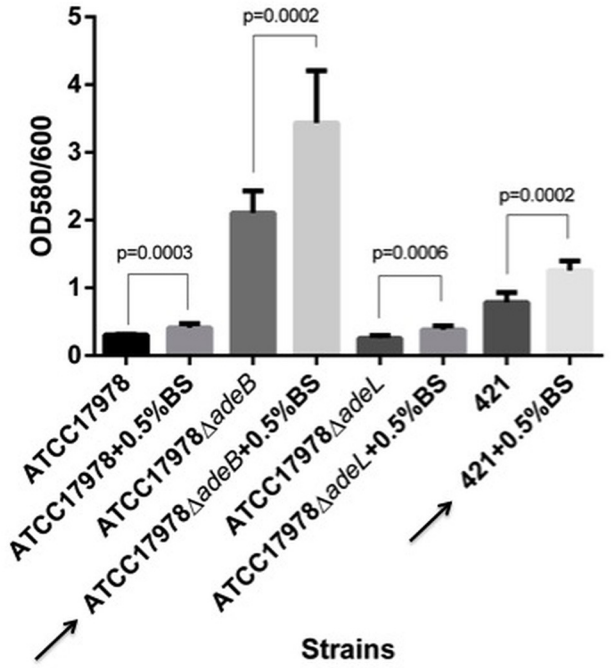


FIGURE 3 | Quantification of biofilm formation by crystal violet staining. Eight independent replicates were considered. Results were analyzed by a Student's *t*-test. The values are means and bars indicate the standard deviation. Arrows indicate the highest biofilm producing strains cultured in the presence of bile salts (BS): *A. baumannii* Δ*adeB* ATCC 17978 and Ab421 GEIH-2010.

TABLE 2 | Microarray analysis of the expression of genes isolated from *Acinetobacter baumanni* isogenic strains cultured in the presence of bile salts (0.5%).

Gene name	Protein Description	Fold	Function
***Acinetobacter baumannii* ATCC 17978Δ*adeL* vs. *Acinetobacter baumannii* ATCC 17978**			
A1S_2303	LysR regulator family	2.50	Regulatory
***Acinetobacter baumannii* ATCC 17978Δ*adeB* vs. *Acinetobacter baumannii* ATCC 17978**			
A1S_1490	Glutamate/Aspartate transporter	1.50	Acid tolerance
A1S_0658	Transposase (ISAba1)	2.57	Mobility of genes
A1S_0657	Transposase (ISAba2)	2.21	
A1S_2218	CsuA/B	1.76	Surface motility/biofilm
A1S_1071	Hypothetical protein	1.88	–
A1S_2652	Hypothetical protein	1.88	
A1S_3020	Hypothetical protein	1.84	
Acinetobacter baumannii* ATCC 17978Δ*adeB* vs. *Acinetobacter baumannii* ATCC 17978Δ*adeL			
A1S_1493	Glutamate/aspartate transport protein	1.60	Acid tolerance
A1S_1490	Glutamate/aspartate transport protein	1.59	
A1S_1492	Glutamate/aspartate transport protein	1.58	
A1S_0113	Acyl-CoA dehydrogenase	1.73	Quorum sensing
A1S_0112	Acyl-CoA synthetase/AMP-acid ligases II	1.65	
A1S_0115	Amino acid adenylation	1.52	
A1S_0114	Acyl carrier protein	1.50	
A1S_0116	RND superfamily transporter	1.50	
A1S_1860	Ring hydroxylating dioxygenase Rieske (2Fe-2S)	1.54	Iron/Sulfur metabolism
A1S_1859	Aromatic-ring-hydroxylating dioxygenase ß subunit[a]	1.50	
A1S_0658	Transposase (ISAba1)	2.74	Mobility of genes
A1S_0657	Transposase (ISAba2)	2.41	
A1S_1294	Type VI secretion system-associated protein (VipA)	2.05	T6SS
A1S_1296	Type VI secretion system-associated protein (Hcp-1)	1.79	
A1S_1292	Putative signal peptide	1.64	
A1S_1295	Type VI secretion system-associated protein (Putative Membrane protein)	1.62	
A1S_1293	Type VI secretion system-associated protein (VipB)	1.99	
A1S_2218	CsuA/B	1.57	Surface motility/biofilm
A1S_1510	Fimbrial protein (type I)	1.55	
A1S_1865	Glu-tRNA amidotransferase	1.64	Transferase activity
A1S_1466	Glutaminase-aspirginase	1.58	
A1S_1071	Hypothetical protein	1.92	–
A1S_2652	Hypothetical protein	1.91	
A1S_3020	Hypothetical protein	1.80	

transporter and associated proteins in tolerance to physiological concentrations of bile salts (Prouty et al., 2004; Sánchez et al., 2005).

Virulence factors associated with activation of the QS system may be regulated differently throughout the intestine depending on the bile salts present, the level of tolerance and the presence of different commensal bacteria (Bachmann et al., 2015). We observed overexpression of the QS genes (A1S_0112 to A1S_0116) in strains lacking the AdeABC efflux pump (*A. baumannii* Δ*adeB* ATCC 17978 and clinical strains of ST79/PFGE-HUI-1) cultured in the presence of bile salts. In a study using transcriptomic analysis, Clemmer et al. confirmed that this cluster of genes was induced by the 3-OH C12-HSL molecule (a signal in the QS network). Interestingly, the A1S_0116 gene encodes a RND superfamily transporter which may be involved in efflux of the molecules from the QS system (Clemmer et al., 2011). Moreover, virulence factors modulated by the QS system were overexpressed, i.e., surface motility, biofilm formation (A1S_2218 and A1S_1510) (Clemmer et al., 2011; Rumbo-Feal et al., 2013) and the Type VI Secretion System (T6SS) (A1S_1292 to A1S_1296) (Sana et al., 2012).

Zheng et al., investigated the role of the QS system in *Vibrio cholerae* in modulating the expression of virulence factors such as T6SS (Zheng et al., 2010). The authors established that a high density of bacteria is critical for expression of the T6SS pandemic *V. cholerae* C6706. The T6SS may be a potent mediator of survival of the pathogen or commensals in multi-bacterial environments, biofilms and in polymicrobial

TABLE 3 | Microarray analysis of the expression of genes isolated from *Acinetobacter baumanni* isogenic strains cultured in the presence (0.5%) and absence of bile salts.

Gene name	Protein Description	Fold	Function
***Acinetobacter baumannii* ATCC 17978 vs. *Acinetobacter baumannii* ATCC 17978 (0.5 % Bile Salts)**			
A1S_3175	Bacterioferritin	5.21	Ferric iron binding
A1S_0800	Bacterioferritin	2.71	
A1S_1860	Ring hydroxylating dioxygenase Rieske (2Fe-2S) protein	2.27	Iron/Sulfur metabolism
A1S_1859	Aromatic-ring-hydroxylating dioxygenase beta subunit	2.21	
A1S_2102	Aldehyde dehydrogenase 1	3.57	Oxidoreductase activity
A1S_1864	Acyl-CoA dehydrogenase-like protein	2.34	
A1S_1858	Short-chain dehydrogenase/reductase SDR	2.17	
A1S_1865	Glu-tRNA amidotransferase	2.66	Transferase activity
A1S_3415	Maleylacetoacetate isomerase	2.5	Isomerase activity
A1S_1857	Vanillate O-demethylase oxidoreductase	2.31	Catalytic activity
A1S_3414	Fumarylacetoacetase	2.14	Fumarylacetoacetate activity
A1S_2809	Bacteriolytic lipoprotein entericidin B	2.03	Response to toxic substance
A1S_1228	Cold shock protein	2	DNA binding
A1S_1924	Cytochrome d terminal oxidase polypeptide subunit I	2.34	Component of membrane
***Acinetobacter baumannii* ATCC 17978Δ*adeB* vs. *Acinetobacter baumannii* ATCC 17978Δ*adeB* (0.5% Bile Salts)**			
A1S_3175	Bacterioferritin	4.17	Ferric iron binding
A1S_0800	Bacterioferritin	3.64	
A1S_1860	Ring hydroxylating dioxygenase Rieske (2Fe-2S) protein	2.79	Iron/Sulfur Metabolism
A1S_1859	Aromatic-ring-hydroxylating dioxygenase beta subunit	2.73	
A1S_1861	Benzoate dioxygenase large subunit	2.43	
A1S_2102	Aldehyde dehydrogenase 1	2.39	Oxidoreductase activity
A1S_1864	Acyl-CoA dehydrogenase-like protein	2.79	
A1S_1858	Short-chain dehydrogenase/reductase SDR	2.53	
A1S_1075	D-amino-acid dehydrogenase	2.62	
A1S_1856	P-hydroxyphenylacetate hydroxylase C1:reductase component	2.01	
A1S_1865	Glu-tRNA amidotransferase	2.97	Transferase activity
A1S_3415	Maleylacetoacetate isomerase	2.59	Isomerase activity
A1S_1857	Vanillate O-demethylase oxidoreductase	2.47	Catalytic activity
A1S_3414	Fumarylacetoacetase	2.12	Fumarylacetoacetate activity
A1S_0804	Trehalose-6-phosphate phosphatase	3.66	Metal ion binding
A1S_1498	TetR family transcriptional regulator	2.28	Transcriptional regulator
A1S_1687	Transcriptional regulator		
A1S_3416	Glyoxalase/bleomycin resistance protein/dioxygenase	2.11	Dioxygenase activity
A1S_1773	RND family drug transporter	2.04	Integral component membrane
A1S_1228	Cold shock protein	4.27	DNA binding
***Acinetobacter baumannii*ATCC 17978Δ*adeL* vs. *Acinetobacter baumannii* ATCC 17978Δ*adeL* Bile Salts (0.5% Bile Salts)**			
A1S_3175	Bacterioferritin	7.63	Ferric iron binding
A1S_0800	Bacterioferritin	5.5	
A1S_2102	Aldehyde dehydrogenase 1	2.59	Oxidoreductase activity
A1S_1865	Glu-tRNA amidotransferase	2.26	Transferase activity
A1S_3415	Maleylacetoacetate isomerase	3.9	Isomerase activity
A1S_3317	Putative outer membrane protein	2.68	Component of membrane
A1S_3414	Fumarylacetoacetase	2.89	Fumarylacetoacetate activity
A1S_2809	Bacteriolytic lipoprotein entericidin B	2.76	Response to toxic substance
A1S_1687	Transcriptional regulator	2.68	Transcriptional regulator
A1S_3416	Glyoxalase/bleomycin resistance protein/dioxygenase	3.17	Dioxygenase activity
A1S_1962	Recombinase A	2.84	DNA metabolism
A1S_1224	Curved DNA-binding protein	2.16	
A1S_1228	Cold shock protein	4.92	
A1S_1031	DNA-binding ATP-dependent protease La	2.75	ATP binding
A1S_1030	DNA-binding ATP-dependent protease La	2.26	
A1S_1950	Putative universal stress protein	2.36	Response to stress
A1S_0364	Transposase	2.18	Mobility of genes
A1S_0683	Putative sigma (54) modulation protein RpoX	2.12	General metabolism
A1S_1987	Putative UDP-galactose 4-epimerase (GalE-like)	2.12	

infections, such as those encountered in the airways of cystic fibrosis patients (Rumbo-Feal et al., 2013; Bachmann et al., 2015) and in gastrointestinal colonization in these patients (Bachmann et al., 2015). Three T6SS systems (H1, H2, and H3-T6SS) with functions at different stages of the infection process (colonization vs. dissemination) or the infection mode (acute virulence vs. chronic persistence) have been described in *Pseudomonas aeruginosa* strain PAO1 (Jani and Cotter, 2010; Schwarz et al., 2010). Moreover, H2-T6SS is regulated by QS in this pathogen (Sana et al., 2012).

Several studies have investigated the role of the T6SS system in *A. baumannii* strains. Repizo et al., reported the first case of *A. baumannii* environmental strain DSM30011 in which the T6SS system was implicated in host colonization (Repizo et al., 2015). Recently, Weber and collaborators demonstrated expression of the T6SS system in *A. baumannii* clinical strains susceptible to several antibiotics (Weber et al., 2015). The authors explained that T6SS is an antibacterial system used by

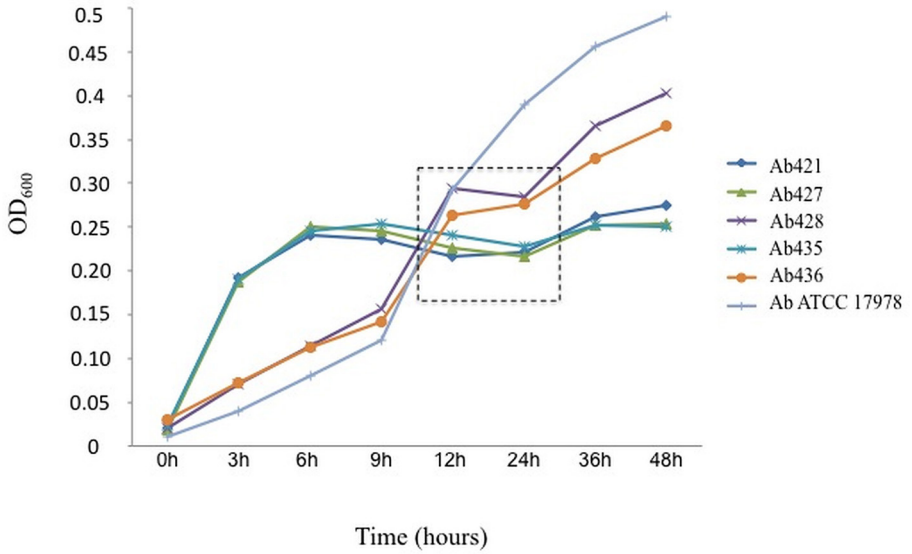


FIGURE 4 | Curves showing growth of the *A. baumannii* isolates belonging to clone ST79/PFGE-HUI-1 in the presence of bile salts (0.5%) in LB-LN. *A. baumannii* ATCC 17978 was included as a control.

TABLE 4 | Expression of genes involved in Acid tolerance, Quorum sensing, T6SS, and surface motility/biofilm mechanisms in clinical strains (clone ST79/PFGE-HUI) cultured in the presence of bile salts (0.5%), determined by RT-PCR.

***A.baumannii* GEIH-2010 (PFGE-HUI-1 clone)**	**Acid tolerance (*A1S_1490* gene[a])**	**Quorum sensing (*A1S_0115* gene[a])**	**T6SS (*A1S_1295* gene[a])**	**Surface motility/biofilm (*A1S_1510* gene[a])**	**Bacteraemia**
Ab421	4.16	4.28	3.38	3.83	Yes
Ab427	2.42	1.68	2.28	2.42	Yes
Ab428	2.60	1.87	2.36	2.41	Yes
Ab435	9.00	9.78	7.83	8.39	Yes
Ab436	4.72	2.80	3.27	4.31	Yes
Control*	**Acid tolerance (*A1S_1490* gene[a])**	**Quorum sensing (*A1S_0115* gene[a])**	**T6SS (*A1S_1295* gene[a])**	**Surface motility/biofilm (*A1S_1510* gene[a])**	
A. baumannii ATCC 17978	1.23	1.03	1.10	1.05	–

**A. baumannii ATCC 17978 was included as a control. The same grown in the absence of bile salts were used as references strains (RE = 1). [a]Genome A. baumannii ATCC 17978.*

Gram-negative bacteria to kill competitors. The *A. baumanni* clinical strains carry T6SS repressors (TetR regulators) in plasmids harboring resistance genes that prevent expression of these T6SS systems (Weber et al., 2015, 2016). When Multi-Drug-Resistant (MDR) strains of *A. baumannii* are not exposed to antibiotics, such as in the inanimate hospital environment or in untreated polymicrobial infections, there is an increased likelihood of encountering competitors that will activate bacterial T6SS systems (Weber et al., 2015). In Spanish hospitals, ST79/PFGE-HUI-1 was the only carbapenem-susceptible clone that did not possess a plasmid carrying OXA 24 ß-lactamase or the AbKAB Toxin-Antitoxin system (Rumbo et al., 2013; Mosqueda et al., 2014). In the present study, resistance to antimicrobials (aminoglycosides, quinolones and glycines) in Ab421 GEIH-2010 (belonging to ST79/PFGE-HUI-1) and *A. baumannii* Δ*adeL* ATCC 17978 was associated with overexpression of the AdeFGH pump.

Finally, although only a small number of cases were considered (out to seven), all patients from whom strains of *A. baumannii* clone ST79/PFGE-HUI-1 were isolated had infections and five out of patients developed bacteraemia (72%). This contrasts with usual observations for *A. baumannii*, as about half of colonized patients do not usually have infections due to the pathogen (Cisneros and Rodríguez-Baño, 2002; Villar et al., 2014), and the prevalence of bacteraemia in infected patients is usually lower than 10% (Thom et al., 2010). Thom and collaborators found that in 86% of patients from ICUs who had the gastrointestinal tract colonized by *A. baumannii* clinical strains, they had bacteraemia through genetically similar strains (Thom et al., 2010). This implies

that those clinical isolates of *A. baumannii* that present a higher capacity to survive gastrointestinal conditions (including bile salts tolerance) through biofilm formation and others mechanisms as transporters could present an increase in their invasive capacity (development of bacteraemia) due to virulence factors (as the type VI secretion system) previously activated under pressure conditions.

In conclusion, this is the first study about the adaptive response to bile salts investigating the molecular and microbiological characteristics in response to bile salts of an isogenic model of *A. baumannii* ATCC 17978 (*A. baumannii* Δ*adeB* ATCC 17978) and clinical isolates of *A. baumannii* (clinical strains of ST79/PFGE-HUI-1) lacking the main RND efflux pump (AdeABC). The response to bile salts led to activation of the QS system and modulated virulence factors such as surface motility, biofilm and Type VI secretion system. Moreover, we observed a new clinical profile (increased invasiveness) of strains of *A. baumannii* ST79/PFGE-HUI-1 lacking the AdeABC efflux pump. Further studies should be carried out with clinical strains of *A. baumannii* with or without the AdeABC efflux pump (or in which the pump is inhibited by treatment) (Pannek et al., 2006; Blair and Piddock, 2009; López et al., 2014; Richmond et al., 2016) under others stress conditions (oxidative and osmotic) that may activate global mechanisms such as the QS system, which modulates virulence factors.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The genome sequence of Ab421 GEIH-2010 strain has been deposited at GenBank under accession number CP014266.1. This genome sequence was determined as part of a II Spanish multicenter study, GEIH-REIPI *A. baumannii* 2000–2010 project (PRJNA308422).

AUTHOR CONTRIBUTIONS

Funding acquisition: MT; Investigation: ML, LB, EG, LF, LM, FF, JR, AP (4th author), AP (9th author), GB, and MT; Methodology: ML, LB, EG, LF; Supervision: MT; Writing: MT.

FUNDING

This study was funded by grant PI13/02390 and PI16/01163 awarded to MT within the State Plan for R+D+I 2013-2016 (National Plan for Scientific Research, Technological Development and Innovation 2008-2011) and co-financed by the ISCIII-Deputy General Directorate of evaluation and Promotion of Research—European Regional Development Fund "A way of Making Europe" and Instituto de Salud Carlos III FEDER, Spanish Network for Research in Infectious Diseases (REIPI RD12/0015). MT was financially supported by the Miguel Servet Research Programme (C.H.U.A Coruña and ISCIII).

ACKNOWLEDGMENTS

We are grateful to the following organizations and researchers who participated in the study: Virgen Rocío (Jerónimo Pachón, Jose Miguel Cisneros, José Garnacho, Antonio Gutierrez-Pizarraya, Juan Antonio Márquez-Vácaro, Younes Smani); Hospital Marqués de Valdecilla (María Eliecer Cano, M. Carmen Fariñas); Hospital Clinic (Ignasi Roca, Paula Espinal); Hospital SAS La Línea (Antonio Sánchez-Porto, Gloria Esteban Meruendano, Luis Barbeyto-Vales, Javier Casas-Ciria, Luis Vallejo); Complejo hospitalario de Ourense (Begona Fernández-Pérez, José Carlos Villar-Chao); Hospital Gregorio Maranón (Belén Padilla-Ortega, Emilia Cercenado-Mansilla); Hospital de Navarra (José Javier García-Irure); Hospital Costa del Sol-Marbella (Alfonso del Arco Jiménez); Hospital General de Valencia (Concepción Gimeno-Cardona, Juan Carlos Valía, Núria Tormo-Palop, Vicente Abril, Josefina Rifa, Maria Jesus Martinez-Garcia); Consorci Hospitalari de Vic (Joseph Vilaró-Pujals, Marian Navarro Aguirre, Ana Vilamala); Policlínica Guipúzkoa (José Antonio Jiménez-Alfaro, Carlos Reviejo-Jaca); Hospital Puerta del Mar (Pilar Marín Casanova, Francisca Guerreo, Evelyn Shaw, Virginia Plasencia); Complejo Hospitalario de Soria (Teresa Nebreda-Mayoral, María José Fernández-Calavia, Susana García de Cruz, Carmen Aldea-Mansilla); Hospital Universitario de Alicante (Esperanza Merino de Lucas, Alfredo Zorraquino, Sergio Reus-Bañuls); Hospital Infanta Cristina (Eugenio Garduno-Eseverri, Luis López Sánchez); Hospital Universitario Central de Asturias (Ana Fleites-Gutiérrez, Azucena Rodríguez-Guardado, Alfonso Moreno); Hospital Donostia (José María García-Arenzana Anguera); Complejo Hospitalario Torrecárdenas (Serafín López-Palmero, Manuel Rodríguez-Maresca); Complejo Hospitalario Xeral-Calde Lugo (Fernando García-Garrote, José Varela-Otero, María del Pilar Alonso); Hospital Universitario Reina Sofía de Córdoba (Elisa Vidal-Verdú, Fernando Rodríguez-López); Hospital Universitario Santiago Compostela (Fernanda Pardo-Sánchez, E. Ferrer-Vizoso, B. Regueiro-Garcia); Hospital Sant Pau (Mercé Gurgui, Roser Pericas,Virginia Pomar); Hospital Galdakao-Usansolo (Pedro María Olaechea-Astigarraga, Rafael Ayarza-Igartua); Hospital Son Dureta (María Dolores Maciá-Romero, Enrique Ruiz de Gopegui-Bordes); Hospital Puerta de Hierro (María Isabel Sánchez-Romero); Hospital Juan Grande (Jesús García-Mata, María José Goyanes, Cristina Morales-Mateos); Hospital San Cecilio (José Hernández-Quero, Trinidad Escobar-Lara); Hospital Sant Joan de Reus (Frederic Ballester-Bastardie, Simona Iftimie, Isabel Pujol-Bajador); Hospital de Motril (María Isabel Galán-Navarro, María Luz Cádiz-Gurrea); Hospital San Agustín (Carmen Amores-Antequera, Montserrat Gómez,Purificación Cantudo); Hospital de Granollers (Carmina Martí-Salas, Jordi Cuquet-Peragosa, Antonio Moreno-Flores, Luis Anibarro-García); Hospital de Segovia (Susana Hernando-Real, Pablo A. Carrero-González); Complejo Hospitalario de Pontevedra (María Angeles Pallarés-González, Sergio Rodríguez-Fernández); Hospital de Bellvitge (Miquel Pujol-Rojo, Fe Tubau); Hospital Virgen de la Victoria de Málaga (Enrique Nuno-Alvarez, María Ortega-Torres); Hospital Doctor Moliner (Salvador Giner-Almaraz, María Rosa Roca-Castelló, Manuela

Castillo, Elena Hortelano); Hospital 12 de Octubre (Fernando Chaves-Sánchez, Ana García-Reyne); Hospital delMar (Juan Pablo Horcajada-Gallego, Concha Segura); Hospital San Agustín de Avilés (Gema Sierra-Dorado, Raquel Yano-Escudero); Complejo Hospitalario Materno Insular de Gran Canaria (María Elena Dorta-Hung, Cristóbal del Rosario Q).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00143/full#supplementary-material

Table S1 | Primers and probes used in this study (Supplementary material).

REFERENCES

Álvarez-Fraga, L., Pérez, A., Rumbo-Feal, S., Merino, M., Vallejo, J. A., Ohneck, E. J., et al. (2016). Analysis of the role of the LH92_11085 gene of a biofilm hyper-producing *Acinetobacter baumannii* strain on biofilm formation and attachment to eukaryotic cells *Virulence* 7, 443–455. doi: 10.1080/21505594.2016.1145335

Antunes, L. C., Visca, P., and Towner, K. J. (2013). *Acinetobacter baumannii*: evolution of a global pathogen. *Pathog. Dis.* 71, 292–301. doi: 10.1111/2049-632X.12125

Aranda, J., Poza, M., Shingu-Vázquez, M., Cortés, P., Boyce, J. D., Adler, B., et al. (2013). Identification of a DNA-damage-inducible regulon in *Acinetobacter baumannii*. *J. Bacteriol.* 195, 5577–5582. doi: 10.1128/JB.00853-13

Bachmann, V., Kostiuk, B., Unterweger, D., Diaz-Satizabal, L., Ogg, S., and Pukatzki, S. (2015). Bile salts modulate the mucin-activated type VI secretion system of pandemic *Vibrio cholerae*. *PLoS Negl. Trop. Dis.* 9:e0004031. doi: 10.1371/journal.pntd.0004031

Beceiro, A., Tomás, M., and Bou, G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin. Microbiol. Rev.* 26, 185–230. doi: 10.1128/CMR.00059-12

Blair, J. M., and Piddock, L. J. (2009). Structure, function and inhibition of RND efflux pumpsin gram-negativebacteria: an update. *Curr. Opin. Microbiol.* 12, 512–519. doi: 10.1016/j.mib.2009.07.003

Cefai, C., Richards, J., Gould, F. K., and McPeake, P. (1990). An outbreak of *Acinetobacter* respiratory tract infection resulting from incomplete disinfection of ventilatory equipment. *J. Hosp. Infect.* 15, 177–182. doi: 10.1016/0195-6701(90)90128-B

Chu, Y. W., Chau, S. L., and Houang, E. T. (2006). Presence of active efflux systems AdeABC, AdeDE and AdeXYZ in different *Acinetobacter* genomic DNA groups. *J. Med. Microbiol.* 55(Pt 4), 477–478. doi: 10.1099/jmm.0.46433-0

Cisneros, J. M., and Rodríguez-Baño, J. (2002). Nosocomial bacteremia due to *Acinetobacter baumannii*: epidemiology, clinical features and treatment. *Clin. Microbiol. Infect.* 8, 687–693. doi: 10.1046/j.1469-0691.2002.00487.x

Clemmer, K. M., Bonomo, R. A., and Rather, P. N. (2011). Genetic analysis of surface motility in *Acinetobacter baumannii*. *Microbiology* 157(Pt 9), 2534–2544. doi: 10.1099/mic.0.049791-0

CLSI, Clinical and Laboratory Standards Institute (2015). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement (M100-S25).*

Corbella, X., Pujol, M., Ayats, J., Sendra, M., Ardanuy, C., Domínguez, M. A., et al. (1996). Relevance of digestive tract colonization in the epidemiology of nosocomial infections due to multiresistant *Acinetobacter baumannii*. *Clin. Infect. Dis.* 23, 329–334. doi: 10.1093/clinids/23.2.329

Coyne, S., Courvalin, P., and Perichon, B. (2011). Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrob. Agents Chemother.* 55, 947–953. doi: 10.1128/AAC.01388-10

Coyne, S., Rosenfeld, N., Lambert, T., Courvalin, P., and Perichon, B. (2010). Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 54, 4389–4393. doi: 10.1128/AAC.00155-10

del Mar Tomas, M., Cartelle, M., Pertega, S., Beceiro, A., Llinares, P., Canle, D., et al. (2005). Hospital outbreak caused by a carbapenem-resistant strain of *Acinetobacter baumannii*: patient prognosis and risk-factors for colonisation and infection. *Clin. Microbiol. Infect.* 11, 540–546. doi: 10.1111/j.1469-0691.2005.01184.x

Gaddy, J. A., Tomaras, A. P., and Actis, L. A. (2009). The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect. Immun.* 77, 3150–3160. doi: 10.1128/IAI.00096-09

Hamad, M. A., Zajdowicz, S. L., Holmes, R. K., and Voskuil, M. I. (2009). An allelic exchange system for compliant genetic manipulation of the select agents *Burkholderia pseudomallei* and *Burkholderia mallei*. *Gene* 430, 123–131. doi: 10.1016/j.gene.2008.10.011

Hamner, S., McInnerney, K., Williamson, K., Franklin, M. J., and Ford, T. E. (2013). Bile salts affect expression of *Escherichia coli* O157:H7 genes for virulence and iron acquisition, and promote growth under iron limiting conditions. *PLoS ONE* 10:e74647. doi: 10.1371/journal.pone.0074647

He, X., Lu, F., Yuan, F., Jiang, D., Zhao, P., Zhu, J., et al. (2015). Biofilm formation caused by clinical *Acinetobacter baumannii* isolates is associated with overexpression of the AdeFGH Efflux pump. *Antimicrob. Agents Chemother.* 59, 4817–4825. doi: 10.1128/AAC.00877-15

Hood, M. I., Jacobs, A. C., Sayood, K., Dunman, P. M., and Skaar, E. P. (2010). *Acinetobacter baumannii* increases tolerance to antibiotics in response to monovalent cations. *Antimicrob. Agents Chemother.* 54, 1029–1041. doi: 10.1128/AAC.00963-09

Jani, A. J., and Cotter, P. A. (2010). Type VI secretion: not just for pathogenesis anymore. *Cell Host Microbe* 8, 2–6. doi: 10.1016/j.chom.2010.06.012

Jin, L. Z., Ho, Y. W., Abdullah, N., and Jalaludin, S. (1998). Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. *Lett. Appl. Microbiol.* 27, 183–185. doi: 10.1046/j.1472-765X

Krastel, K., Senadheera, D. B., Mair, R., Downey, J. S., Goodman, S. D., and Cvitkovitch, D. G. (2010). Characterization of a glutamate transporter operon, glnQHMP, in *Streptococcus mutans* and its role in acid tolerance. *J. Bacteriol.* 192, 984–993. doi: 10.1128/JB.01169-09

Lin, J., Sahin, O., Michel, L. O., and Zhang, Q. (2003). Critical role of multidrug efflux pump CmeABC in bile resistance and *in vivo* colonization of *Campylobacter jejuni*. *Infect. Immun.* 71, 4250–4259. doi: 10.1128/IAI.71.8.4250-4259.2003.

Lin, L., Ling, B. D., and Li, X. Z. (2009). Distribution of the multidrug efflux pump genes, adeABC, adeDE and adeIJK, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex. *Int. J. Antimicrob. Agents* 33, 27–32. doi: 10.1016/j.ijantimicag.2008.06.027

Lopez, M., Álvarez-Fraga, L., Gato, E., Blasco, L., Poza, M., Fernández-García, L., et al. (2016). Genome sequence of a clinical strain of *Acinetobacter baumannii* belonging to the ST79/PFGE-HUI-1 clone lacking the AdeABC (resistance-nodulation-cell division-type) efflux pump. *Genome Announc.* 4:e00962-16. doi: 10.1128/genomeA.00962-16

López, M., Barbosa, B., Gato, E., Bou, G., and Tomas, M. (2014). Patents on antivirulence therapies. *World J. Pharmacol.* 3, 97–109. doi: 10.5497/WJP.v3.i4.97

López, M., Mayer, C., Fernández-García, L., Blasco, L., Muras, A., Ruiz, F. M., et al. (2017). Quorum sensing network in clinical strains of *A. baumannii*: AidA is a new quorum quenching enzyme. *PLoS ONE* 12:e0174454. doi: 10.1371/journal.pone.0174454

Malik, N. A. (2016). Solubilization and interaction studies of bile salts with surfactants and drugs: a review. *Appl. Biochem. Biotechnol.* 179, 179–201. doi: 10.1007/s12010-016-1987-x

Mosqueda, N., Gato, E., Roca, I., López, M., de Alegría, C. R., Fernández Cuenca, F., et al. (2014). Characterization of plasmids carrying the blaOXA-24/40 carbapenemase gene and the genes encoding the AbkA/AbkB proteins of a toxin/antitoxin system. *J. Antimicrob. Chemother.* 69, 2629–2633. doi: 10.1093/jac/dku179

Mulin, B., Talon, D., Viel, J. F., Vincent, C., Leprat, R., Thouverez, M., et al. (1995). Risk factors for nosocomial colonization with multiresistant *Acinetobacter baumannii*. *Eur. J. Clin. Microbiol. Infect. Dis.* 14, 569–576. doi: 10.1007/BF01690727

Pannek, S., Higgins, P. G., Steinke, P., Jonas, D., Akova, M., Bohnert, J. A., et al. (2006). Multidrug efflux inhibition in *Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-beta-naphthylamide. *J. Antimicrob. Chemother.* 57, 970–974. doi: 10.1093/jac/dkl081

Prouty, A. M., Brodsky, I. E., Falkow, S., and Gunn, J. S. (2004). Bile-salt-mediated induction of antimicrobial and bile resistance in *Salmonella typhimurium*. *Microbiology* 150(Pt 4), 775–783. doi: 10.1099/mic.0.26769-0

Pumbwe, L., Skilbeck, C. A., and Wexler, H. M. (2008). Presence of quorum-sensing systems associated with multidrug resistance and biofilm formation in *Bacteroides fragilis*. *Microb. Ecol.* 56, 412–419. doi: 10.1007/s00248-007-9358-3

Pumbwe, L., Skilbeck, C. A., Nakano, V., Avila-Campos, M. J., Piazza, R. M., and Wexler, H. M. (2007). Bile salts enhance bacterial co-aggregation, bacterial-intestinal epithelial cell adhesion, biofilm formation and antimicrobial resistance of *Bacteroides fragilis*. *Microb. Pathog.* 43, 78–87. doi: 10.1016/j.micpath.2007.04.002

Repizo, G. D., Gagné, S., Foucault-Grunenwald, M. L., Borges, V., Charpentier, X., Limansky, A. S., et al. (2015). Differential role of the T6SS in *Acinetobacter baumannii* virulence. *PLoS ONE* 10:e0138265. doi: 10.1371/journal.pone.0138265

Rey, I., Soubigou, P., Debussche, L., David, C., Morgat, A., Bost, P. E., et al. (1989). Antibodies to synthetic peptide from the residue 33 to 42 domain of c-Ha-ras p21 block reconstitution of the protein with different effectors. *Mol. Cell. Biol.* 9, 3904–3910. doi: 10.1128/MCB.9.9.3904

Richmond, G. E., Evans, L. P., Anderson, M. J., Wand, M. E., Bonney, L. C., Ivens, A., et al. (2016). The *Acinetobacter baumannii* two-component system AdeRS regulates genes required for multidrug efflux, biofilm formation, and virulence in a strain-specific manner. *MBio* 7:e00852-16. doi: 10.1128/mBio.00852-16

Rodríguez-Baño, J., Cisneros, J. M., Fernández-Cuenca, F., Ribera, A., Vila, J., Pascual, A., et al. (2004).Clinical features and epidemiology of *Acinetobacter baumannii* colonization and infection in Spanish hospitals. *Infect. Control Hosp. Epidemiol.* 25, 819–824. doi: 10.1086/502302

Rumbo, C., Gato, E., López, M., Ruiz de Alegría, C., Fernández-Cuenca, F., Martínez-Martínez, L., et al. (2013). Contribution of efflux pumps, porins, and β-lactamases to multidrug resistance in clinical isolates of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 57, 5247–5257. doi: 10.1128/AAC.00730-13

Rumbo-Feal, S., Gómez, M. J., Gayoso, C., Álvarez-Fraga, L., Cabral, M. P., Aransay, A. M., et al. (2013). Whole transcriptome analysis of *Acinetobacter baumannii* assessed by RNA-sequencing reveals different mRNA expression profiles in biofilm compared to planktonic cells. *PLoS ONE* 8:e72968. doi: 10.1371/journal.pone.0072968

Sana, T. G., Hachani, A., Bucior, I., Soscia, C., Garvis, S., Termine, E., et al. (2012). The second type VI secretion system of *Pseudomonas aeruginosa* strain PAO1 is regulated by quorum sensing and Fur and modulates internalization in epithelial cells. *J. Biol. Chem.* 287, 27095–27105. doi: 10.1074/jbc.M112.376368

Sánchez, B., Champomier-Vergès, M. C., Anglade, P., Baraige, F., de Los Reyes-Gavilán, C. G., Margolles, A., et al. (2005). Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. *J. Bacteriol.* 187, 5799–5808. doi: 10.1128/JB.187.16.5799-5808.2005

Schwarz, S., Hood, R. D., and Mougous, J. D. (2010). What is type VI secretion doing in all those bugs? *Trends Microbiol.* 18, 531–537. doi: 10.1016/j.tim.2010.09.001

Sherertz, R. J., and Sullivan, M. L. (1985). An outbreak of infections with *Acinetobacter calcoaceticus* in burn patients: contamination of patients' mattresses. *J. Infect. Dis.* 151, 252–258. doi: 10.1007/BF01643394

Thom, K. A., Hsiao, W. W., Harris, A. D., Stine, O. C., Rasko, D. A., and Johnson, J. K. (2010). Patients with *Acinetobacter baumannii* bloodstream infections are colonized in the gastrointestinal tract with identical strains. *Am. J. Infect. Control* 38, 751–753. doi: 10.1016/j.ajic.2010.03.005

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., and Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology* 149(Pt 12), 3473–3484. doi: 10.1099/mic.0.26541-0

Tomas, M., Doumith, M., Warner, M., Turton, J. F., Beceiro, A., Bou, G., et al. (2010). Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob. Agents Chemother.* 54, 2219–2224. doi: 10.1128/AAC.00816-09

Villar, M., Gato, E., Garnacho-Montero, J., Cisneros, J. M., Ruíz de Alegría, C., Fernández-Cuenca, F., et al. (2014). Epidemiological and clinical impact of Acinetobacter baumannii colonization and infection: a reappraisal. *Medicine* 93, 202–210. doi: 10.1099/mic.0.26541-0

Weber, B. S., Hennon, S. W., Wright, M. S., Scott, N. E., de Berardinis, V., Foster, L. J., et al. (2016). Genetic dissection of the type VI secretion system in *Acinetobacter* and identification of a novel peptidoglycan hydrolase, TagX, required for its biogenesis. *MBio* 7:e01253-16. doi: 10.1128/mBio.01253-16

Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S., and Feldman, M. F. (2015). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proc. Natl. Acad. Sci. U.S.A.* 112, 9442–9447. doi: 10.1073/pnas.1502966112

Yoon, E. J., Chabane, Y. N., Goussard, S., Snesrud, E., Courvalin, P., Dé, E., et al. (2015). Contribution of resistance-nodulation-cell division efflux systems to antibiotic resistance and biofilm formation in *Acinetobacter baumannii*. *MBio* 6:e00309-15. doi: 10.1128/mBio.00309-15

Zheng, J., Shin, O. S., Cameron, D. E., and Mekalanos, J. J. (2010). Quorum sensing and a global regulator TsrA control expression of type VI secretion and virulence in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21128–21133. doi: 10.1073/pnas.1014998107

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 21 June 2017
doi: 10.3389/fcimb.2017.00274

Biofilm-Induced Type 2 Innate Immunity in a Cystic Fibrosis Model of *Pseudomonas aeruginosa*

*Kenny Bielen[1,2], Bart 'S Jongers[1], Jan Boddaert[1], Tom K. Raju[1], Christine Lammens[2], Surbhi Malhotra-Kumar[2], Philippe G. Jorens[3], Herman Goossens[2] and Samir Kumar-Singh[1,2]**

[1] Molecular Pathology Group, Laboratory of Cell Biology and Histology, Faculty of Medicine and Health Sciences, University of Antwerp, Wilrijk, Belgium, [2] Laboratory of Medical Microbiology—Vaccine and Infectious Disease Institute, University of Antwerp, Wilrijk, Belgium, [3] Laboratory Experimental Medicine and Pediatrics, Department of Critical Care Medicine, Antwerp University Hospital and University of Antwerp, Edegem, Belgium

Biofilm-producing strains of *Pseudomonas aeruginosa* are a major cause of morbidity and mortality in cystic fibrosis (CF) patients. In these patients, increased levels of IL-17 as well as of IL-5 and IL-13 along with arginase (Arg)-positive macrophages have been observed in bronchoalveolar lavage fluid. While IL-17 is a strong proinflammatory cytokine associated with host defense against bacterial and fungal infections and is also elevated in several autoimmune diseases, IL-5/IL-13 and Arg1-positive M2 macrophages are part of the anti-inflammatory type 2 (Th2) immunity. To study whether increased IL-5 and IL-13 levels are related to biofilm formation, which is frequently observed in CF patients colonized by *P. aeruginosa*, we utilized an agarose bead-embedded *P. aeruginosa* rat model commonly employed in *in vivo* biofilm studies. We showed that "sterile" agarose bead instillation in rat notably increased lung transcript levels of IL-5 and IL-13 at two post-instillation study-points, day 1 and day 3. Concurrently, increased infiltration of type 2 innate cells such as eosinophils and Arg1 positive M2 activated macrophages (Arg1+CD68+) was also observed both at day 1 and day 3 while the proportion of M1 activated macrophages (iNOS+CD68+) at these time-points decreased. In contrast, *P. aeruginosa*-loaded beads caused a drastic elevation of proinflammatory Th1 (IFNγ, TNFα, IL-12a) and antibacterial Th17 (IL-17a, IL-17f, IL-22, IL-23a) cytokines along with a high influx of neutrophils and M1 macrophages, while Th2 cytokines (IL-5 and IL-13) drastically declined at day 1 post-infection. Interestingly, at day 3 post-infection, both Th1 and Th17 cytokines sharply declined and corroborated with decreased M1 and increased M2 macrophages. These data suggest that while IL-17 is linked to episodes of acute exacerbations of infection in CF patients, the increased Th2 cytokines and M2 macrophages observed in these patients are largely due to the biofilm matrix. The data presented here has important implications for clinical management of CF patients.

Keywords: *P. aeruginosa* pneumonia, chronic pneumonia, animal model, agar beads, M2 macrophage, eosinophil, Th2, Th17

OPEN ACCESS

Edited by:
Ghassan M. Matar, American University of Beirut, Lebanon

Reviewed by:
Thomas Elliott Van Dyke, Forsyth Institute, United States
Balasubramanian Natesan, Universidade Nova de Lisboa, Portugal

***Correspondence:**
Samir Kumar-Singh
samir.kumarsingh@uantwerpen.be

Received: *28 November 2016*
Accepted: *06 June 2017*
Published: *21 June 2017*

Citation:
Bielen K, 'S Jongers B, Boddaert J, Raju TK, Lammens C, Malhotra-Kumar S, Jorens PG, Goossens H and Kumar-Singh S (2017) Biofilm-Induced Type 2 Innate Immunity in a Cystic Fibrosis Model of Pseudomonas aeruginosa. Front. Cell. Infect. Microbiol. 7:274. doi: 10.3389/fcimb.2017.00274

INTRODUCTION

Pseudomonas aeruginosa is the major cause of pulmonary infection in cystic fibrosis (CF) patients (Gibson et al., 2003). Approximately 80% of CF patients become chronically colonized/infected with *P. aeruginosa* by late adolescence and these patients can live with such infections for years (Lyczak et al., 2002). Chronic *P. aeruginosa* colonization is also associated with increased morbidity and mortality in CF patients especially during episodes of acute exacerbations (Bhatt, 2013). This heightened vulnerability and persistence of bacterial colonization is caused by increased mucous secretion in the alveolar spaces that provide an ideal environment to form biofilms (Sadikot et al., 2005; Moreau-Marquis et al., 2008).

Biofilm mainly consists of bacterial-derived exopolysaccharides that protect the encapsulated bacteria from host immune cells and antibiotics. Interestingly, suppressive therapy reduces *P. aeruginosa* lung burden, but does not impact lung biofilm burden (Fernandez-Barat et al., 2016). To mimic this distinct pathology observed in CF and other chronic obstructive lung diseases such as bronchiectasis, agarose beads are employed to establish a chronic lung infection model. In this model, bacteria are slowly released from agarose. Agarose is a linear polymer of repeating units of agarobiose that closely mimics the extracellular polymeric substances or exopolysaccharides secreted by *P. aeruginosa* (Cash et al., 1979; Growcott et al., 2011). Genetically modified murine models based on mutations in gene encoding for cystic fibrosis transmembrane conductance regulator (CFTR) have contributed invaluably to the current understanding of CFTR function. However, the CFTR genetic models lack the development of spontaneous lung pneumonia observed in CF patients (Fisher et al., 2011). Furthermore, the bioelectric characteristics in the tracheal airways of mice and humans diverge significantly and CFTR genetic models continue to demonstrate substantial cAMP-inducible changes in chloride permeability despite the absence of a functioning CFTR (Grubb et al., 1994; Liu et al., 2006).

Chronic *P. aeruginosa* pneumonia occurring in CF patients causes a persistent lung inflammation dominated by neutrophils and an antibody response against *P. aeruginosa* (Moser et al., 2000). Neutrophils and macrophages are the most important first responders within the innate immune system. Specifically, macrophages due to their longer life-spans are the major effector population in the first line of defense against invading pathogens (Serbina et al., 2008; Hanke and Kielian, 2012). This cell type is classically divided into proinflammatory M1 macrophages that are chiefly responsible for bacterial clearance, and anti-inflammatory M2 macrophages important in tissue repair processes. M1 macrophages are commonly identified by their high inducible nitric oxide synthase (iNOS) production and M2 macrophages are identified due to their high arginase (Arg) 1 expression (reviewed in Benoit et al., 2008).

Interestingly, increased levels of anti-inflammatory Th2 cytokines (IL-5 and IL-13) and antibacterial Th17 cytokine (IL-17A) are observed in bronchoalveolar lavage (BAL) fluid of CF patients chronically colonized or infected with *P. aeruginosa* (Moser et al., 2000; Hartl et al., 2006; Tiringer et al., 2013). Several studies have also shown an increase in M2 macrophages and eosinophils in BAL fluid of CF patients who had been *P. aeruginosa* carriers at some stage in their lifetimes (Grasemann et al., 2005; Murphy et al., 2010). While CF patients frequently show a heightened Th2 and Th17 cytokine response at a given stage during the disease, it is not known whether and how these cytokines are linked to *P. aeruginosa* or its biofilm. A skewing of macrophages toward M2 polarization with increased Arg1 and reduced iNOS expression has been evidenced in catheter-associated staphylococcal biofilms (Thurlow et al., 2011). In this study, we investigated the innate immune response to biofilm-like structures in the chronic pneumonia agar bead model and showed that sterile biofilm beads could solely incite the specific type 2 immune response observed in CF patients.

METHODS

Bacterial Dose Preparation

Agar beads were prepared following a previously published protocol with minor modifications (Growcott et al., 2011). Briefly, colonies from an overnight culture of *P. aeruginosa* were diluted with 30 mL sterile PBS to achieve a suspension of 0.3 O.D. at 600 nm. Cultures were washed and the pellets re-suspended in 4 mL sterile PBS. One milliliter of bacterial suspension was added to 10 mL of sterile 2% agar at 52°C and the mixture added to 15 mL heavy mineral oil (52°C) supplemented with 0.02% sorbitan-monooleate (Sigma-Aldrich) and mixed thoroughly. Beads were centrifuged at 10,000 × g at 4°C, extensively washed with sterile PBS and filtered using a 200 μm nylon mesh. Beads were resuspend in 2 × w/v PBS and 500 μL bead suspension (≈ 2E7 colony forming units) was used per animal. Sterile beads were prepared using sterile saline. Each bead solution was plated on blood-agar plates and incubated overnight at 37°C for inoculum validation.

Anesthesia and Endotracheal Intubation

All animal experiments were conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations and approved by the University of Antwerp Ethics Committee. Rats were anesthetized using 100 mg/kg ketamine and 1 mg/kg medetomidine and intubated utilizing a tilting intubation platform (Hallowell EMC). Briefly, anesthetized rats were placed on the tilting platform in a horizontal position. An elastic band was placed behind the front incisors and attached to the platform. Next a 14 G catheter was placed into the trachea with the aid of a guide wire. With 14 G catheter, animals were instilled with agarose beads, extubated, and anesthesia antagonized with 300 μg/kg atipamezole. Animals were placed back in their cages and followed up.

Animal Experimental Groups

A total of 36 adult male Wistar rats (mean weight 340 g, SD = 23 g, Charles River) were used in this study and randomly assigned into one of the five experimental groups: (i) Non-manipulated animals served as the control group (n = 12; no bead group); (ii) Two sterile agarose (St) bead groups euthanized at day 1 and day 3 post-instillation, respectively (n = 6 animals

per group); (iii) Two chronic pneumonia (Pa-bead) groups where animals were instilled with 2×10^7 CFU of *P. aeruginosa* enmeshed in agarose beads and euthanized at day 1 and day 3 post-infection, respectively ($n = 6$ animals per group).

Animal Follow-Up and Euthanasia

Animals were closely monitored for clinical signs of pneumonia (SI Table 1). Euthanasia was performed by isoflurane overdose and blood collected by cardiac puncture utilizing EDTA coated tubes. The trachea was exposed and lungs lavaged using a 14-gauge angiocatheter with 20 mL/kg body-weight of ice-cold sterile PBS. Right lung was removed, washed in sterile PBS and snap frozen in liquid nitrogen. Left lung was washed and fixed overnight in 2% paraformaldehyde and prepared for paraffin embedding.

Histopathology

Lung pathology scoring was performed on H&E stained 5 μm thick paraffin sections, as done previously (Matute-Bello et al., 2011). All lungs used for histology were treated with standardized protocols to allow comparisons between the groups. Lung pathology was assessed blinded by a trained pathologist. Amount of neutrophils for each group were manually counted on 8–10 consecutive images grabbed using 200x magnification per slide for each animal, eosinophils were quantified on 400x magnification. For slides that showed heterogenic staining pattern, most affected areas were imaged for quantification.

Immunohistochemistry

Immunohistochemistry was done as described by us previously (Wils et al., 2012). Briefly, antigen retrieval was performed in citrate buffer (0.018 M citric acid.H_2O and 0.082 M sodium citrate.H_2O) using microwave heating. Endogenous peroxidase was blocked using 0.3% H_2O_2 for 20 min. Blocking of non-specific antigens was done using 1:5 diluted normal horse serum in 1% BSA solution in PBS (PBS-BSA) for 30 min. Primary antibodies were diluted in PBS-BSA solution and incubated overnight at 4°C: anti-CD68, 1:200 dilution (Abd Serotec MCA341R); anti-arginase-1, 1:400 dilution (Santa Cruz Sc-18354); and anti-iNOS, 1:100 (Abcam ab15323). Secondary biotinylated antibodies (Jackson Immunoresearch) were used in 1:200 dilution, incubated for 30 min at room temperature, washed and incubated with extravidin-HRP for 30 min followed by DAB (5', 5' diaminobenzedine, Dako) development. Double-labeled immunohistochemistry was performed using anti-CD68 (1:200 dilution), anti-arginase1 (1:400 dilution), and anti-iNOS (1:100 dilution) with DAG-cy3, DAM-cy5 and DAR-cy5 in 1:200 dilutions. Sections were co-stained with 5 μg/mL DAPI (Sigma-Aldrich) and coverslipped using antifading PBS-glycerol mounting medium (Citifluor).

Light microscopy images were grabbed on Olympus UC30 color camera. Quantification was performed by calculating the percentage of stained area using pixel-by pixel analysis after spectral de-convolution of the image using the IHC profiler plug-in in ImageJ v1.47 (Varghese et al., 2014). The mean percentage of positive stained area per animal was used. Slides were randomized before analyses by a blinded investigator. High-resolution images from double-labeled immunofluorescence stained sections were grabbed using a dual spinning disk confocal microscope (Ultra*View* VoX, PerkinElmer) and images analyzed using Volocity (PerkinElmer). Proportions of Arg1+CD68+ and iNOS+CD68+ cells were manually counted by the blinded investigator.

Lung Transcript Analyses

Total RNA from lung tissue was extracted using RNeasy-mini spin columns (Qiagen) after grinding in liquid nitrogen. RNA integrity and concentrations were estimated using RNA-nanochips on Bio-analyzer (Agilent). Extracted RNA was converted to cDNA by reverse transcriptase (RT^2 First Strand Kit, Qiagen). Quantitative PCR was performed using CFX connect (Bio-Rad) using custom PCR-arrays (Qiagen) for following genes of interest: *IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12a, IL-13, IL-17a, IL-17f, IL-22, IL-23a, Ifnγ, Tnfα*. Housekeeping genes included were: *Ywhag, Actb, Polr2b, Gapdh*, *Sdha,* and *Tbp*. Additionally, a random genomic DNA contamination control, three reverse transcriptase controls, and three additional PCR controls were used. Data was validated on independently extracted RNA and analyzed using in-house primers (primer sequences available on request) using SYBRGreen assay. Data was analyzed using comparative C_T method as described earlier (Kumar-Singh et al., 2006; Schmittgen and Livak, 2008). Briefly, the combined average Ct value of the control group was used to calculate the fold differences in the study groups.

Data Analyses and Statistics

Data analyses were performed using SPSS version 23 (IBM) and presented as either averages or average fold-differences with the standard errors of mean. Kolmogorov-Smirnov test for normality was used for each data set before testing statistical significance of differences on log-transformed values by one-way ANOVA with *post-hoc* Bonferroni corrections. Survival analyses were performed using Kaplan Meier estimator with Mantel-Cox log rank test for testing significant differences. Values of all significant correlations ($P < 0.05$) are given with degree of significance indicated ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

Sterile Biofilm Matrix-Mimicking Agar Beads Induce Th2 Cytokine Expression

To study whether a Th2 response could be provoked by the presence of biofilm matrix, we intratracheally instilled animals with sterile agar beads and analyzed lung transcript levels of key cytokines at day 1 (d1) and day 3 (d3) post-bead instillation time points. Compared to non-manipulated control animals (No bead group), sterile beads did not cause an increase in proinflammatory Th1 cytokine expression of *Ifnγ, Tnfα*, *IL-1β*, IL-12a at d1 post-bead instillation (**Figure 1A**, white bars). Analyzing the lung transcript levels for the Th17 cytokine family, we showed increased expression of *IL-17a* (157-fold, $P < 0.001$) and *IL-22* (112-fold, $P < 0.01$) at d1 post-bead instillation that were sustained at the d3 time-point (*IL-17a*, 158-fold, $P < 0.001$; *IL-22*, 65-fold, $P < 0.01$; **Figure 1B**).

Interestingly, lung transcript levels of Th2 cytokines in sterile agar beads-instilled animals were markedly elevated with

increased expression of *IL-5* and *IL-13* at d1 post-bead instillation (*IL-5,* 39-fold, $P < 0.01$; *IL-13,* 102-fold, $P < 0.001$) that remained elevated at d3 (*IL-5*, 30-fold, $P < 0.05$; *IL-13*, 26-fold, $P < 0.001$). Transcript levels of *IL-4* were not altered by sterile agar-bead instillation (**Figure 1C**). Additionally, elevated transcript levels compared to control group were also noticed for immunomodulatory cytokines *IL-6* (d1, 38-fold, $P < 0.001$; d3, 21-fold, $P < 0.05$) and *IL-10* (d1, 15-fold, $P < 0.05$; d3, 20-fold, $P < 0.05$; **Figure 1D**).

Th2 Innate Cellular Pulmonary Infiltration Induced by "Sterile" Agar Bead Instillation

IL-5 and IL-13 are potent inducers of eosinophil chemotaxis in the lung (Pope et al., 2001) and IL-13 has been shown to be involved with M2 macrophage polarization causing depressed antibacterial immunity (Knippenberg et al., 2015). Therefore, we next studied these two innate type 2 effector cells. Compared to control animals, sterile beads caused a moderate increase in neutrophil infiltration in lung at both time-points ($P < 0.05$ for both), confirming previous observations (Cash et al., 1979; Growcott et al., 2011) (SI Figure 1). Additionally, sterile beads also caused an intense eosinophilic infiltration at both studied time-points ($P < 0.001$ for both; **Figures 2A,B,D**), especially in regions surrounding bronchioles harboring agarose beads.

Similarly, an intense macrophage infiltration was also associated with sterile beads. However, due to overlapping macrophages especially around the agarose beads, an automated quantification estimating percentage area occupation of immunohistochemical staining was employed as described in *Methods*. With this protocol, we showed that CD68+ areas were significantly increased at d1 and d3 (two and five times, respectively), compared to control animals (P at least < 0.05 for both; **Figures 2A,B,D**). This coincided with significantly increased Arg1+ areas at both time-points (P at least < 0.01; **Figures 2B,D**). These data were confirmed on confocal double-labeled immunofluorescence microscopy showing that 31% of activated CD68+ macrophages were Arg1+ already at d1 and this proportion of M2 macrophages increased to 82% at d3 compared to controls ($P < 0.01$ for d1; and $P < 0.001$ for d3 group; **Figures 3A,C**). Additionally, a proportional drop in numbers of M1 macrophages (iNOS+CD68+) was observed compared to controls ($P < 0.001$; **Figures 3B,D**). These data indicate that agarose biofilm structures induce an acute Th2 innate cellular pathology marked by elevated eosinophils and M2 macrophages in rat lung.

High Th17 Cytokine Profile in a *P. aeruginosa* Agar Bead Pneumonia Model

To study how relevant agarose/biofilm-induced Th2/Th17 cytokines are compared to the infectious situation, and whether these Th2 cytokines and Th2 innate cells observed in the sterile bead model persist after *P. aeruginosa* co-challenge, we established a Pa-bead model. In this model, rats were intratracheally instilled with agarose beads enmeshed with *P. aeruginosa* that led to development of progressive pneumonia evident up to d3 post-infection (SI Figure 2A). Pa-bead animals showed ≈50% mortality at d3 post-infection whereas sterile bead animals showed no mortality (Kaplan-Meier, $^{***}P < 0.001$, Mantel-Cox log rank test; SI Figure 2B).

Intratracheal instillation of *P. aeruginosa* enmeshed beads caused a drastic reduction in the observed Th2 response with both *IL-5* and *IL-13* attenuated by up to 20 and 30 times, respectively, compared to sterile bead instilled animals (**Figure 1C**). This coincided with an expected surge at d1 of all studied proinflammatory cytokines (*Ifnγ*, 99-fold; *Tnfα*, 117-fold; *IL-1β*, 82-fold; *IL-12a*, 16-fold; P for all < 0.001; **Figure 1A**). In accordance with earlier studies (Lavoie et al., 2011; Lovewell et al., 2014), a high expression of immunomodulatory cytokines *IL-6* (6,600-fold) and *IL-10* (211-fold; P for both < 0.001) was also observed (**Figure 1D**).

Interestingly, the surge in IL-17 family of cytokines was much more pronounced. At d1 post-infection, *IL-17a* and *IL-17f* were upregulated 25,000 and 15,000-fold, respectively, compared to controls, and ≈20,000 and ≈1,500-fold compared to sterile bead-instilled animals (**Figure 1B**). These cytokines are not only important in mediating neutrophil chemotaxis (Xu et al., 2014), but are also amongst the important drivers of proinflammatory cytokine expression including *IL-1β* and *Tnfα* that we also observed to be elevated in our Pa-bead model. IL-23, another member of the IL-17 family and one of the strongest drivers of Th17 cell differentiation and IL-17 secretion (Bedoya et al., 2013) was also significantly elevated (**Figure 1B**). Lastly, IL-22 that along with IL-17 has important functions in mucosal immunity against extracellular pathogens, particularly Gram-negative organisms (Aujla et al., 2008; Xu et al., 2014) was also elevated by several 100-folds in lung transcripts (**Figure 1B**). However, at d3 post-infection, transcripts of the Th17 family subsided to levels comparable to sterile bead-instilled animals. These data suggest that the IL-17 response observed in CF patients is primarily pathogen-driven.

Increased Arg1+ Activated M2 Macrophages and Eosinophils in *P. aeruginosa* Agar Bead Pneumonia Model

While patient studies have shown an increased arginase activity in BAL fluid of CF patients colonized with *P. aeruginosa* (Grasemann et al., 2005; Murphy et al., 2010), the individual contribution of the pathogen and the biofilm matrix remain unknown. Additionally, as bacteria are one of the most important drivers of M1 macrophage polarization, we studied whether the increased type 2 innate cellular infiltrates induced by sterile agarose beads persist in the Pa-bead model.

At both d1 and d3 time-points, Pa-bead-instilled animals were characterized by high loads of neutrophils compared to controls ($P < 0.01$; SI Figure 1) and to the sterile-bead group ($P < 0.01$ for d1; $P < 0.05$ for d3; SI Figure 1), as shown previously (van Heeckeren and Schluchter, 2002; Growcott et al., 2011). Interestingly, while eosinophilic counts in the Pa-bead group were reduced at d1 and d3 time points (≈3 and ≈5 times, respectively), these were still highly elevated in the Pa-bead group compared to the controls ($P < 0.001$; **Figures 2C,D**). These data are also consistent with sporadic reports of increased eosinophil activation in lung of CF patients with *P. aeruginosa* pneumonia (Koller et al., 1994).

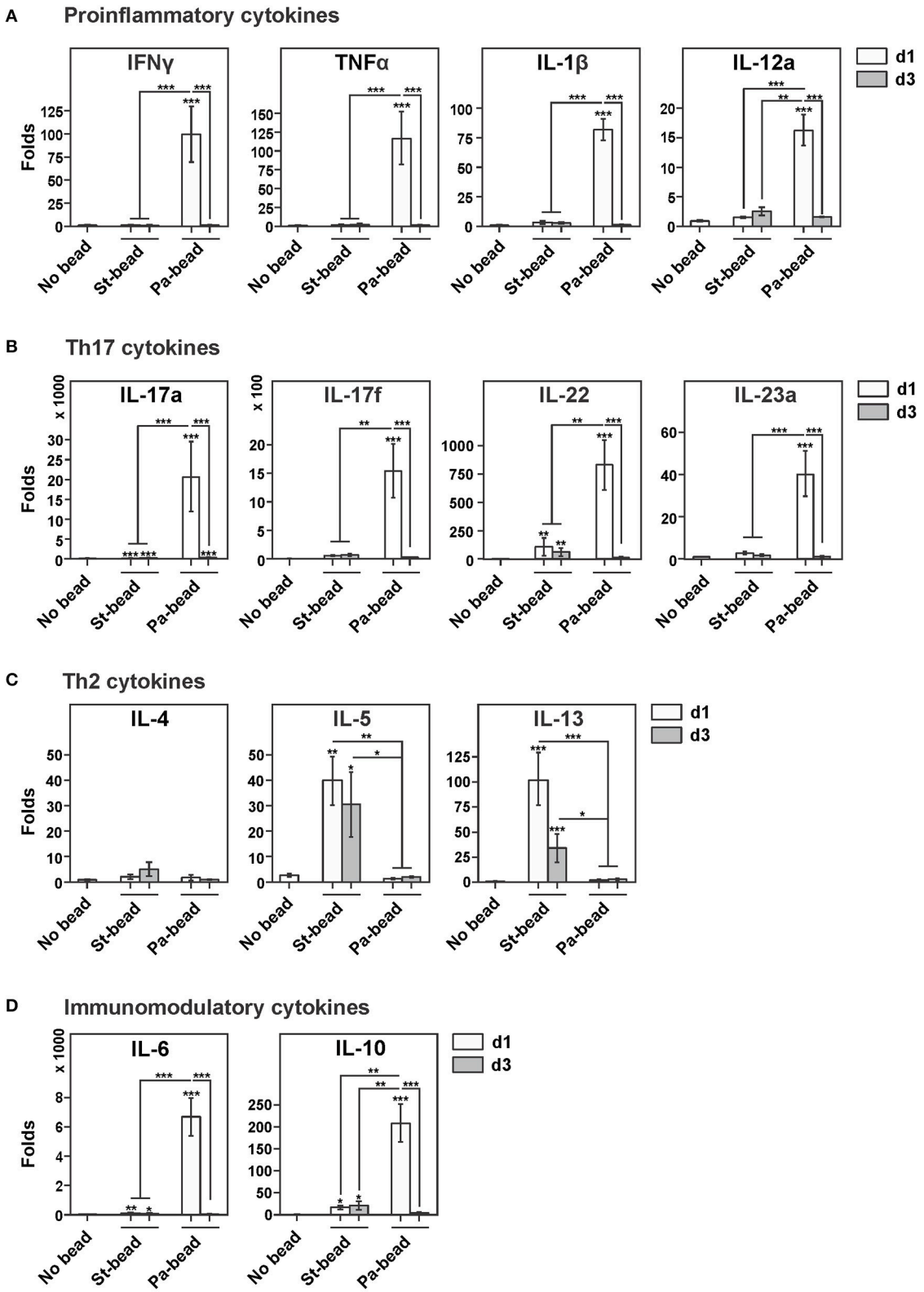


FIGURE 1 | Lung transcript levels of key cytokines in response to intratracheal instillation in rats of sterile agar beads or *P. aeruginosa*-loaded beads **(A)**. Lung transcript levels of main proinflammatory cytokines *Ifnγ*, *Tnfα*, *IL-1β,* and *IL-12a* for sterile beads (St-bead) and for *P. aeruginosa* beads (Pa-bead) at post-infection

(Continued)

FIGURE 1 | Continued
time-points day (d)1 (white columns) and d3 (gray columns), compared to control animals (No bead). The most drastic elevation for these cytokines was noted for Pa-bead group at d1. **(B)** Similarly, Th17 cytokine analysis of *IL-17a, IL-17f, IL22*, *IL23a* showed highest lung expression levels in Pa-bead group at d1 post-infection. **(C)** Increased expression of Th2 cytokines *IL-5* and *IL-13* was noted for St-bead group at both d1 and d3 time-points. **(D)** Highest lung expression levels of immunomodulatory cytokines *IL-6* and *IL-10* for Pa-bead group at d1. **(A–D)**, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; asterisk above the vertical bars denotes significance against No bead control group; $n = 6$ animals per group.

Importantly, we showed that the Pa-bead group had ≈3-fold increased lung infiltration of CD68+ cells at both d1 and d3 time-points compared to the sterile bead group ($P < 0.001$; **Figures 2C,D**). Studying the M1/M2 macrophage markers, we further showed that iNOS+ cells were drastically elevated in the Pa-bead group especially at d1 (d1, 19 times and d3, 5 times, $P < 0.01$ for both), while Arg1+ cells showed a non-significant increase in infiltration in the Pa-bead model at both time-points compared to sterile bead-inoculated animals (≈3 times for both; **Figures 2C,D**). Double-labeled immunofluorescence microscopy confirmed these data showing that at d1, 65% of CD68+ activated macrophages were iNOS+ M1 macrophages ($P < 0.001$; **Figures 3B,D**), while the population of Arg1+ M2 macrophages was 18% and non-significantly elevated compared to controls (**Figures 3B,C**). Moreover, at d3, the Arg1+ M2 macrophage proportion increased to 63% of the total activated macrophage population ($P < 0.001$; **Figures 3B,C**) while iNOS+ M1 macrophages declined to 18% ($P < 0.001$; **Figures 3B,D**). These data indicate that chronic *P. aeruginosa* infection can sustain the anti-inflammatory M2 cellular innate immune response induced by biofilm matrix-mimicking agarose bead-instillation.

DISCUSSION

We showed here that instillation of "sterile" agarose beads in a rat biofilm model, extensively used in the field of cystic fibrosis research, activates the same Th2 cytokines (IL-5 and IL-13) and Th2 innate cells (eosinophils and Arg1+ M2 macrophages) as observed in human CF patients (Moser et al., 2000; Grasemann et al., 2005; Murphy et al., 2010; Tiringer et al., 2013). Interestingly, co-challenge with *P. aeruginosa* caused an acute drastic elevation of Th1/Th17 cytokine transcripts in our model, however, expression of these cytokines declined rapidly by d3. While the precise reason for this drastic decline is not known, it should be noted that animals with the most severe pathology did not survive up to the d3 time-point and this could in part have an influence here. Nevertheless, a decline in Th1/Th17 at d3 corroborated with a drastic drop in M1 macrophages in lungs with a notable increase in the Arg1+CD68+ M2 macrophage population with ≈60% of all activated macrophages being of the M2 phenotype by this time point. Although the upstream driver of this M2 polarization is so far undefined, our data suggest that the Th2/M2 response observed in CF patients is primarily due to biofilm matrix structures rather than a direct effect of the accompanying pathogen. This suggestion also fits well with the observation that pathogens most frequently associated with CF or with other chronic pneumonia pathologies, such as *P. aeruginosa*, *S. aureus,* and *B. cepacia* (Coutinho et al., 2008), have a high biofilm producing capacity.

Similar to human CF patients showing increased IL-5 and IL-13 but not IL-4 levels (Hauber et al., 2003; Tiringer et al., 2013), we also observed only a non-significant increase in IL-4. While IL4 is a key Th2 cytokine, eosinophil-specific IL-13 expression, but not IL-4, has been identified as a major factor for inducing allergic Th2 pulmonary pathologies (Jacobsen et al., 2015). Moreover, increased M2 macrophage infiltration in lung is observed in IL-13-associated lung diseases (Wu et al., 2015) and IL-13 has been shown to independently enhance the M2 macrophage phenotype *in vitro* (Doyle et al., 1994). Similarly, IL-5 on its own is also one of the major chemoattractants for eosinophils (Pope et al., 2001), that, together with M2 macrophages, constitute the two major innate Th2-type effector cells. Interestingly, we also showed in our model a high expression of IL-10, which similar to IL-13 can directly enhance the M2 macrophage phenotype (Makita et al., 2015).

Important for therapeutic implications, and more central to our findings here, increased expression of IL-13 and IL-5 in lungs of CF patients is associated with occurrence of acute pneumonia exacerbations (Tiringer et al., 2013). Moreover, IL-13 induced M2 macrophages can cause reduced antibacterial immunity (Knippenberg et al., 2015). Recent data also suggest that development of acute exacerbations of infection is not caused by the acquisition of new strains but rather a clonal expansion of existing strains Stenbit and Flume, 2011). Perhaps, the development of an anti-inflammatory Th2/M2 environment in the lung with lowered anti-bacterial Th1/M1 immunity in response to biofilm, that we describe here, results in a local immunosuppressive milieu that eventually aids bacterial survival and dispersal. Worryingly, one fourth of all CF patients developing an acute exacerbation do not recover to baseline within 3 months after starting treatment with antibiotics, especially those antibiotics that are linked with increased biofilm formation (Hoffman et al., 2005; Bhatt, 2013). Our data thus indicate that the upregulated Th2/M2 pathway identified here could be a potential drug target used as an antibiotic adjuvant to treat episodes of acute exacerbations in CF patients. In addition, high IL-13 and IL-5 expression could also be used to detect patients at risk of developing acute exacerbations of infection.

We also showed here in a well-described animal model of CF that the acute anti-*P. aeruginosa* immune responses are especially modulated by the IL-17 family of cytokines, members of which are IL-17A, IL-17F, IL-22, and IL-23. High lung transcript levels of all of these cytokines along with an intense migration in the lung of neutrophils and of iNOS+CD68+ M1 macrophages were observed as a direct response toward the pathogen. Although the IL-17 cytokine family is associated with cells of the adaptive

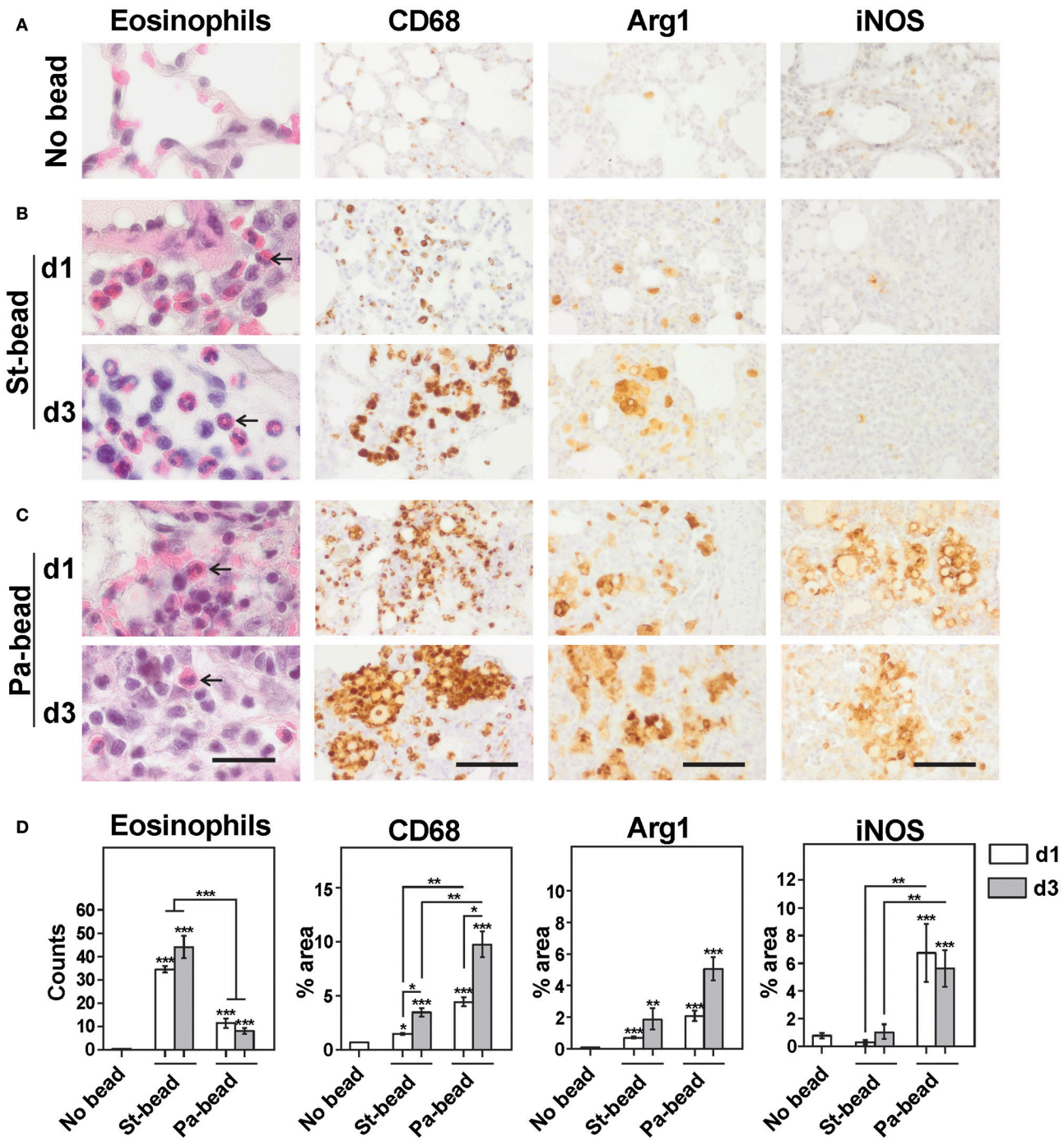


FIGURE 2 | Cellular infiltrates in sterile beads and CF rat model. Representative images for H&E and immunohistochemistry for CD68, Arg1 and iNOS for **(A)** No bead control group, **(B)** Sterile bead group (St-bead) and **(C)** *P. aeruginosa* bead group (Pa-bead). An increased influx of eosinophils was observed in St-bead group but also to a limited extent in the Pa-bead group (arrows). **(D)** Quantitative analyses for the histochemical and immunohistochemical stains. Data in **(D)** is presented as averages ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and asterisk above the vertical bars denotes significance against No bead control group; $n = 6$ animals per group. **(A–C)**, scale bar represents 20 μm for H&E stained and 60 μm for immunostained sections.

arm of the immune system, in bacterial infections such as those caused by *P. aeruginosa*, innate immune cells are the first responders in secretion of IL-17, IL-21, IL-22, and IL-23 (Nieuwenhuis et al., 2002; Aujla et al., 2008; Coquet et al., 2008; Sutton et al., 2012). In this respect, both NKT cells and γδ-T cells have been identified as major sources of IL-17 production

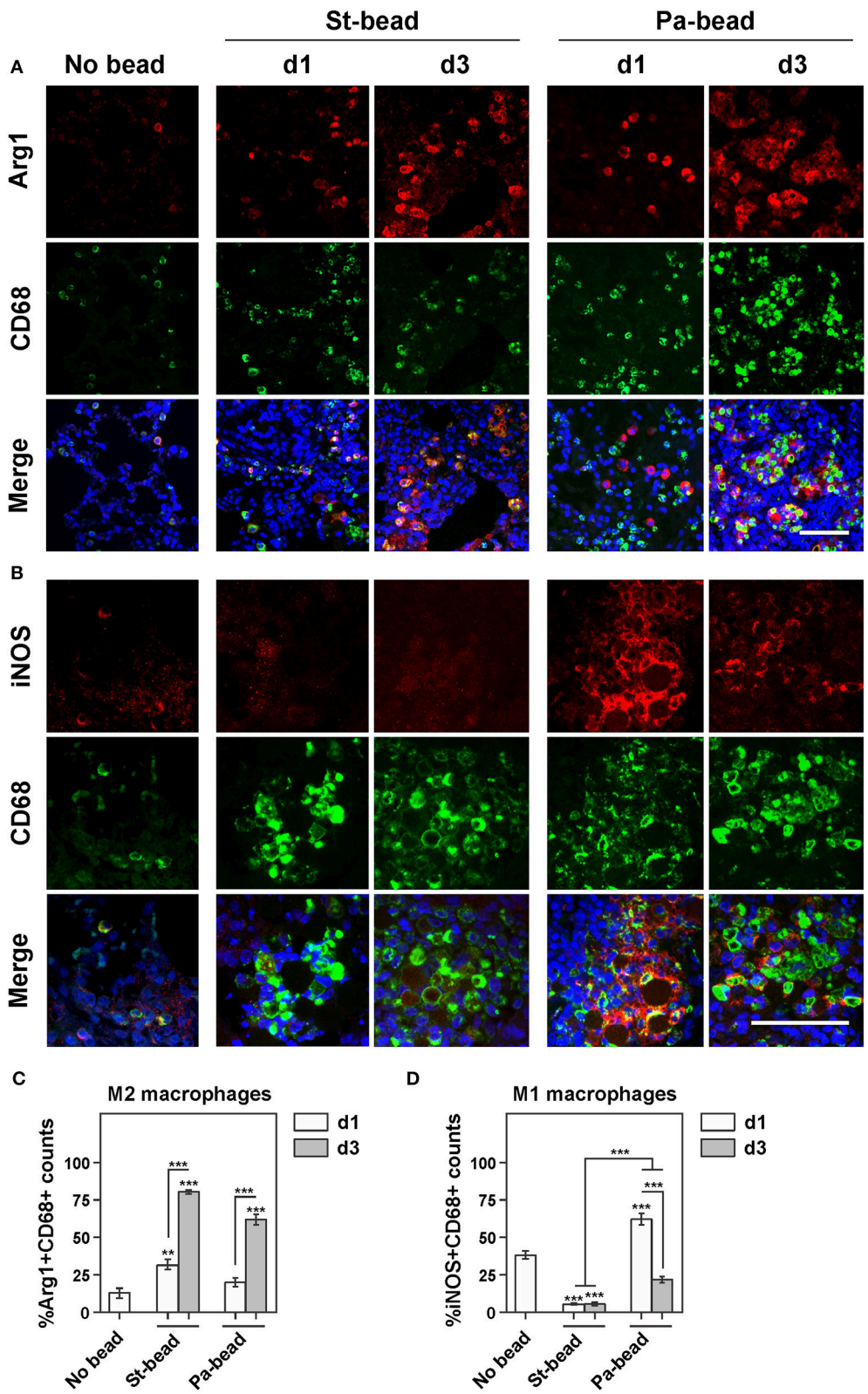


FIGURE 3 | Increased M2 macrophage infiltration following bead instillation **(A)**. Representative images of double-labeled immunohistochemistry for Arg1+CD68+ cells (M2 macrophages) for control (No bead), sterile beads (St-bead), and *P. aeruginosa* beads (Pa-bead) group. **(B)** Representative images of double-labeled

(Continued)

FIGURE 3 | Continued
immunohistochemistry for iNOS+CD68+ cells (M1 macrophages) for these three groups. Quantitative analyses of **(C)** M2 cells (Arg1+CD68+) and **(D)** M1 cells (iNOS+CD68+) presented as average percentage of total CD68+ cell population ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$. Asterisk above the vertical bars denotes significance against No bead control group. $n = 6$ animals per group. Scale bars in **(A,B)** represents 30 μm (iNOS immunoreactivity was assessed at a higher magnification).

at mucosal surfaces, providing protection against *P. aeruginosa* (Nieuwenhuis et al., 2002; Coquet et al., 2008; Sutton et al., 2012), and NK cells have been shown to secrete IL-22 following Gram-negative pneumonia (Aujla et al., 2008). However, the observed antibacterial response mediated by IL-17 family of cytokines was transient and diminished rapidly. As discussed, the majority of activated macrophages were of the M2 phenotype at d3, indicating the initiation of a healing response or an anti-inflammatory response likely mediated by the biofilm-matrix. We stipulate that the precise timing for this switch in the CF agarose model would depend on the absolute and relative amounts of *P. aeruginosa* and agarose matrix challenge.

To conclude, we showed here that the Th2-mediated anti-inflammatory milieu observed in CF patients is primarily driven by the biofilm matrix structures and not by the accompanying pathogen, i.e., *P. aeruginosa*. These data prompt considering the use of IL-5 and IL-13 as biomarkers to detect and treat impending infections in CF patients. We also showed that the acute IL-17 superexpression is directly linked to episodes of acute exacerbations of *P. aeruginosa* infection in rat. These data, we believe, have important implications for the clinical management of CF patients.

AUTHOR CONTRIBUTIONS

SK designed and supervised the study. KB, B'S, JB, and CL carried out the experiments and analyzed data. SK performed the pathological analysis. KB, PJ, SM, HG, and SK interpreted the data. KB and SK wrote the manuscript. All authors read, edited, and approved the final draft.

FUNDING

This study was funded by the Flemish Fund for Scientific Research (FWO-G051312N), Flemish Institute for Science and technology (IWT), and University of Antwerp (GOA-s30729). KB (IWT-SB111664) and B'S (FWO- SB151525) are PhD fellows of FWO/IWT-Belgium.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00274/full#supplementary-material

REFERENCES

Aujla, S. J., Chan, Y. R., Zheng, M., Fei, M., Askew, D. J., Pociask, D. A., et al. (2008). IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat. Med.* 14, 275–281. doi: 10.1038/nm1710

Bedoya, S. K., Lam, B., Lau, K., and Larkin, J. III. (2013). Th17 cells in immunity and autoimmunity. *Clin. Dev. Immunol.* 2013:986789. doi: 10.1155/2013/986789

Benoit, M., Desnues, B., and Mege, J. L. (2008). Macrophage polarization in bacterial infections. *J. Immunol.* 181, 3733–3739. doi: 10.4049/jimmunol.181.6.3733

Bhatt, J. M. (2013). Treatment of pulmonary exacerbations in cystic fibrosis. *Eur. Respir. Rev.* 22, 205–216. doi: 10.1183/09059180.00006512

Cash, H. A., Woods, D. E., McCullough, B., Johanson, W. G. Jr., and Bass, J. A. (1979). A rat model of chronic respiratory infection with *Pseudomonas aeruginosa*. *Am. Rev. Respir. Dis.* 119, 453–459.

Coquet, J. M., Chakravarti, S., Kyparissoudis, K., McNab, F. W., Pitt, L. A., McKenzie, B. S., et al. (2008). Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11287–11292. doi: 10.1073/pnas.0801631105

Coutinho, H. D., Falcao-Silva, V. S., and Goncalves, G. F. (2008). Pulmonary bacterial pathogens in cystic fibrosis patients and antibiotic therapy: a tool for the health workers. *Int. Arch. Med.* 1:24. doi: 10.1186/1755-7682-1-24

Doyle, A. G., Herbein, G., Montaner, L. J., Minty, A. J., Caput, D., Ferrara, P., et al. (1994). Interleukin-13 alters the activation state of murine macrophages *in-vitro* - comparison with Interleukin-4 and interferon-gamma. *Eur. J. Immunol.* 24, 1441–1445. doi: 10.1002/eji.1830240630

Fernandez-Barat, L., Ciofu, O., Kragh, K. N., Pressler, T., Johansen, U., Motos, A., et al. (2016). Phenotypic shift in *Pseudomonas aeruginosa* populations from cystic fibrosis lungs after 2-week antipseudomonal treatment. *J. Cyst. Fibros.* 16, 222–229. doi: 10.1016/j.jcf.2016.08.005

Fisher, J. T., Zhang, Y., and Engelhardt, J. F. (2011). Comparative biology of cystic fibrosis animal models. *Methods Mol. Biol.* 742, 311–334. doi: 10.1007/978-1-61779-120-8_19

Gibson, R. L., Burns, J. L., and Ramsey, B. W. (2003). Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 168, 918–951. doi: 10.1164/rccm.200304-505SO

Grasemann, H., Schwiertz, R., Matthiesen, S., Racke, K., and Ratjen, F. (2005). Increased arginase activity in cystic fibrosis airways. *Am. J. Respir. Crit. Care Med.* 172, 1523–1528. doi: 10.1164/rccm.200502-253OC

Growcott, E. J., Coulthard, A., Amison, R., Hardaker, E. L., Saxena, V., Malt, L., et al. (2011). Characterisation of a refined rat model of respiratory infection with *Pseudomonas aeruginosa* and the effect of ciprofloxacin. *J. Cyst. Fibros.* 10, 166–174. doi: 10.1016/j.jcf.2010.12.007

Grubb, B. R., Paradiso, A. M., and Boucher, R. C. (1994). Anomalies in ion transport in CF mouse tracheal epithelium. *Am. J. Physiol.* 267(1 Pt 1), C293–C300.

Hanke, M. L., and Kielian, T. (2012). Deciphering mechanisms of staphylococcal biofilm evasion of host immunity. *Front. Cell. Infect. Microbiol.* 2:62. doi: 10.3389/fcimb.2012.00062

Hartl, D., Griese, M., Kappler, M., Zissel, G., Reinhardt, D., Rebhan, C., et al. (2006). Pulmonary T(H)2 response in *Pseudomonas aeruginosa*-infected patients with cystic fibrosis. *J. Allergy Clin. Immunol.* 117, 204–211. doi: 10.1016/j.jaci.2005.09.023

Hauber, H. P., Gholami, D., Koppermann, G., Heuer, H. E., Meyer, A., and Pforte, A. (2003). Increased expression of Interleukin-13 but not Interleukin-4 in cystic fibrosis patients. *J. Cyst. Fibros.* 2, 189–194. doi: 10.1016/S1569-1993(03)00091-2

Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A., and Miller, S. I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436, 1171–1175. doi: 10.1038/nature 03912

Jacobsen, E. A., Doyle, A. D., Colbert, D. C., Zellner, K. R., Protheroe, C. A., LeSuer, W. E., et al. (2015). Differential activation of airway eosinophils induces IL-13-mediated allergic Th2 pulmonary responses in mice. *Allergy* 70, 1148–1159. doi: 10.1111/all.12655

Knippenberg, S., Brumshagen, C., Aschenbrenner, F., Welte, T., and Maus, U. A. (2015). Arginase 1 activity worsens lung-protective immunity against *Streptococcus pneumoniae* infection. *Eur. J. Immunol.* 45, 1716–1726. doi: 10.1002/eji.201445419

Koller, D. Y., Gotz, M., Eichler, I., and Urbanek, R. (1994). Eosinophilic activation in cystic fibrosis. *Thorax* 49, 496–499. doi: 10.1136/thx.49.5.496

Kumar-Singh, S., Theuns, J., Van Broeck, B., Pirici, D., Vennekens, K., Corsmit, E., et al. (2006). Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. *Hum. Mutat.* 27, 686–695. doi: 10.1002/humu.20336

Lavoie, E. G., Wangdi, T., and Kazmierczak, B. I. (2011). Innate immune responses to *Pseudomonas aeruginosa* infection. *Microbes Infect.* 13, 1133–1145. doi: 10.1016/j.micinf.2011.07.011

Liu, X., Yan, Z., Luo, M., and Engelhardt, J. F. (2006). Species-specific differences in mouse and human airway epithelial biology of recombinant adeno-associated virus transduction. *Am. J. Respir. Cell Mol. Biol.* 34, 56–64. doi: 10.1165/rcmb.2005-0189OC

Lovewell, R. R., Patankar, Y. R., and Berwin, B. (2014). Mechanisms of phagocytosis and host clearance of *Pseudomonas aeruginosa*. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 306, L591–L603. doi: 10.1152/ajplung.00335.2013

Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* 15, 194–222. doi: 10.1128/CMR.15.2.194-222.2002

Makita, N., Hizukuri, Y., Yamashiro, K., Murakawa, M., and Hayashi, Y. (2015). IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eosinophil migration. *Int. Immunol.* 27, 131–141. doi: 10.1093/intimm/dxu090

Matute-Bello, G., Downey, G., Moore, B. B., Groshong, S. D., Matthay, M. A., Slutsky, A. S., et al. (2011). An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *Am. J. Respir. Cell Mol. Biol.* 44, 725–738. doi: 10.1165/rcmb.2009-0210ST

Moreau-Marquis, S., Stanton, B. A., and O'Toole, G. A. (2008). *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. *Pulm. Pharmacol. Ther.* 21, 595–599. doi: 10.1016/j.pupt.2007.12.001

Moser, C., Kjaergaard, S., Pressler, T., Kharazmi, A., Koch, C., and Hoiby, N. (2000). The immune response to chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients is predominantly of the Th2 type. *APMIS* 108, 329–335. doi: 10.1034/j.1600-0463.2000.d01-64.x

Murphy, B. S., Bush, H. M., Sundareshan, V., Davis, C., Hagadone, J., Cory, T. J., et al. (2010). Characterization of macrophage activation states in patients with cystic fibrosis. *J. Cyst. Fibros.* 9, 314–322. doi: 10.1016/j.jcf.2010.04.006

Nieuwenhuis, E. E., Matsumoto, T., Exley, M., Schleipman, R. A., Glickman, J., Bailey, D. T., et al. (2002). CD1d-dependent macrophage-mediated clearance of *Pseudomonas aeruginosa* from lung. *Nat. Med.* 8, 588–593. doi: 10.1038/nm0602-588

Pope, S. M., Brandt, E. B., Mishra, A., Hogan, S. P., Zimmermann, N., Matthaei, K. I., et al. (2001). IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. *J. Allergy Clin. Immunol.* 108, 594–601. doi: 10.1067/mai.2001.118600

Sadikot, R. T., Blackwell, T. S., Christman, J. W., and Prince, A. S. (2005). Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am. J. Respir. Crit. Care Med.* 171, 1209–1223. doi: 10.1164/rccm.200408-1044SO

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Serbina, N. V., Jia, T., Hohl, T. M., and Pamere, E. G. (2008). Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.* 26, 421–452. doi: 10.1146/annurev.immunol.26.021607.090326

Stenbit, A. E., and Flume, P. A. (2011). Pulmonary exacerbations in cystic fibrosis. *Curr. Opin. Pulm. Med.* 17, 442–447. doi: 10.1097/mcp.0b013e32834 b8c04

Sutton, C. E., Mielke, L. A., and Mills, K. H. (2012). IL-17-producing gammadelta T cells and innate lymphoid cells. *Eur. J. Immunol.* 42, 2221–2231. doi: 10.1002/eji.201242569

Thurlow, L. R., Hanke, M. L., Fritz, T., Angle, A., Aldrich, A., Williams, S. H., et al. (2011). *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation *in vivo*. *J. Immunol.* 186, 6585–6596. doi: 10.4049/jimmunol.1002794

Tiringer, K., Treis, A., Fucik, P., Gona, M., Gruber, S., Renner, S., et al. (2013). A Th17- and Th2-skewed cytokine profile in cystic fibrosis lungs represents a potential risk factor for *Pseudomonas aeruginosa* infection. *Am. J. Respir. Crit. Care Med.* 187, 621–629. doi: 10.1164/rccm.201206-1150OC

van Heeckeren, A. M., and Schluchter, M. D. (2002). Murine models of chronic *Pseudomonas aeruginosa* lung infection. *Lab. Anim.* 36, 291–312. doi: 10.1258/002367702320162405

Varghese, F., Bukhari, A. B., Malhotra, R., and De, A. (2014). IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS ONE* 9:e96801. doi: 10.1371/journal.pone.0096801

Wils, H., Kleinberger, G., Pereson, S., Janssens, J., Capell, A., Van Dam, D., et al. (2012). Cellular ageing, increased mortality and FTLD-TDP-associated neuropathology in progranulin knockout mice. *J. Pathol.* 228, 67–76. doi: 10.1002/path.4043

Wu, K., Byers, D. E., Jin, X., Agapov, E., Alexander-Brett, J., Patel, A. C., et al. (2015). TREM-2 promotes macrophage survival and lung disease after respiratory viral infection. *J. Exp. Med.* 212, 681–697. doi: 10.1084/jem.20141732

Xu, X., Shao, B., Wang, R., Zhou, S., Tang, Z., Lu, W., et al. (2014). Role of Interleukin-17 in defense against *Pseudomonas aeruginosa* infection in lungs. *Int. J. Clin. Exp. Med.* 7, 809–816.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 22 February 2017
doi: 10.3389/fcimb.2017.00045

Colistin Resistance in *Acinetobacter baumannii* MDR-ZJ06 Revealed by a Multiomics Approach

*Xiaoting Hua[1,2], Lilin Liu[1,2], Youhong Fang[3], Qiucheng Shi[1,2], Xi Li[4], Qiong Chen[5], Keren Shi[1,2], Yan Jiang[1,2], Hua Zhou[6] and Yunsong Yu[1,2,7]**

[1] Department of Infectious Diseases, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [2] Key Laboratory of Microbial Technology and Bioinformatics of Zhejiang Province, Hangzhou, China, [3] The Children's Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [4] Centre of Laboratory Medicine, Zhejiang Provincial People's Hospital, Hangzhou, China, [5] Hangzhou First People's Hospital, Hangzhou, China, [6] Department of Respiratory, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [7] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China

Acinetobacter baumannii has emerged as an important opportunistic pathogen due to its ability to acquire resistance to most currently available antibiotics. Colistin is often considered as the last line of therapy for infections caused by multidrug-resistant *A. baumannii* (MDRAB). However, colistin-resistant *A. baumannii* strain has recently been reported. To explore how multiple drug-resistant *A. baumannii* responded to colistin resistance, we compared the genomic, transcriptional and proteomic profile of *A. baumannii* MDR-ZJ06 to the induced colistin-resistant strain ZJ06-200P5-1. Genomic analysis showed that *lpxC* was inactivated by IS*Aba1* insertion, leading to LPS loss. Transcriptional analysis demonstrated that the colistin-resistant strain regulated its metabolism. Proteomic analysis suggested increased expression of the RND efflux pump system and down-regulation of FabZ and β-lactamase. These alterations were believed to be response to LPS loss. In summary, the *lpxC* mutation not only established colistin resistance but also altered global gene expression.

Keywords: *Acinetobacter baumannii*, colistin, whole-genome sequencing, transcriptome, proteome

OPEN ACCESS

Edited by:
Ghassan M. Matar, American University of Beirut, Lebanon

Reviewed by:
Rahul Raghavan, Portland State University, USA
Alice Lebreton, École Normale Supérieure, France

****Correspondence:***
Yunsong Yu
yvys119@163.com

Received: *10 November 2016*
Accepted: *07 February 2017*
Published: *22 February 2017*

Citation:
Hua X, Liu L, Fang Y, Shi Q, Li X, Chen Q, Shi K, Jiang Y, Zhou H and Yu Y (2017) Colistin Resistance in Acinetobacter baumannii MDR-ZJ06 Revealed by a Multiomics Approach. Front. Cell. Infect. Microbiol. 7:45. doi: 10.3389/fcimb.2017.00045

INTRODUCTION

Acinetobacter baumannii has emerged as an important opportunistic pathogen due to its ability to acquire resistance to most currently available antibiotics (Peleg et al., 2008; Howard et al., 2012; Antunes et al., 2014). Since current treatment options for multi-drug resistant (MDR) *A. baumannii* are extremely limited, colistin is often considered as the last line of the therapy for infections caused by MDR *A. baumannii* (Bae et al., 2016; Cheah et al., 2016b). However, colistin-resistant *A. baumannii* strain has recently been reported (Cai et al., 2012).

Colistin is a polycationic antimicrobial peptide that targets the polyanionic bacterial lipopolysaccharide (LPS) of Gram-negative bacteria. Two different colistin resistance mechanisms have previously been reported (Beceiro et al., 2014). The first mechanism inactivates the lipid A biosynthesis pathway, leading to the complete loss of surface LPS. Mutations in *lpxC*, *lpxA*, or *lpxD* are involved in the first mechanism. The *pmrAB* two-component system mediates the second resistance mechanism. Mutations in *pmrA* and *pmrB* induce the activity of *pmrC*, which adds

phosphoethanolamine (PEtn) to the hepta-acylated form of lipid A (Beceiro et al., 2011). Further mutations in *vacJ*, *pldA*, *ttg2C*, *pheS* and a conserved hypothetical protein were reported to involve in reduced colistin susceptibility through novel resistance mechanisms (Thi Khanh Nhu et al., 2016). Four putative colistin resistant genes: *A1S_1983*, *hepA*, *A1S_3026,* and *rsfS* were also identified in our previous study (Mu et al., 2016).

The response to LPS alteration has been investigated via transcriptional analysis. In response to LPS alteration, *A. baumannii* alters the expression of critical transport and biosynthesis systems associated with modulating the composition and structure of the bacterial surface (*lpxA*; Henry et al., 2012) or alters the expression of genes associated with outer membrane structure and biogenesis (*pmrB*; Cheah et al., 2016a). Moreover, the response to colistin is highly similar to the transcriptional alteration observed in an LPS-deficient strain (Henry et al., 2015). Colistin resistance was also explored using proteomic methods. There were 35 differentially expressed proteins. Most differentially expressed proteins were down-regulated in the colistin resistant strain, including outer membrane proteins, chaperones, protein biosynthesis factors, and metabolic enzymes (Fernandez-Reyes et al., 2009). However, the combination of genomic, transcriptomic, and proteomic methods to examine the colistin resistance mechanism in *A. baumannii* has rarely been reported. Furthermore, the strain used in this study was an MDR strain, but not laboratory strains (ATCC 19606, ATCC 17978) that do not represent clonal lineages in a clinical environment. Here, we used genome, transcriptome, and proteome to elucidate the colistin resistance mechanism in MDR *A. baumannii*. There was an ISAba1 insertion in *lpxC* (ABZJ_03720) in ZJ06-200P5-1 compared with the genome sequence of MDR-ZJ06, where *lpxC* encoded an UDP-3-O-acyl-N-acetylglucosamine deacetylase.

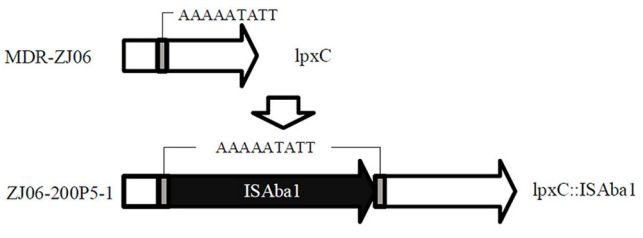


FIGURE 1 | Whole genome sequencing revealed the colistin-resistance mechanism in *A. baumannii* ZJ06-200P5-1. The gene *lpxC* was intact in MDR-ZJ06, while in ZJ06-200P5-1, *lpxC* was inactivated by the insertion sequence ISAba1.

MATERIALS AND METHODS

Bacterial Strains, Media, and Antibiotics

Restriction enzymes, T4 ligase, and Taq DNA polymerase were purchased from TaKaRa (Otsu, Shiga, Japan). The *A. baumannii* strain MDR-ZJ06 was isolated from the bloodstream of a patient in Hangzhou, China, in 2006. All *A. baumannii* cultures were grown at 37 °C in Mueller-Hinton (MH) agar and cation-adjusted MH broth (CAMHB) (Oxoid, Basingstoke, UK). Colistin was purchased from Sigma (Shanghai, China).

Generation of Colistin-Resistant Mutant

A colistin-resistant mutant was generated in *A. baumannii* MDR-ZJ06 by a previously described method (Li et al., 2006). Briefly, first, MDR-ZJ06 was cultured in CAMHB containing colistin at 8 × minimum inhibitory concentration (MIC). After overnight incubation, the culture was diluted 1:1000 with CAMHB containing colistin at 64 × MIC and then incubated at 37 °C overnight. Finally, the culture was diluted 1:100 with CAMHB containing colistin at 200 × MIC. After overnight incubation, the culture was plated on plates containing 10 μg of colistin at an appropriate dilution, and then one of colistin resistant colonies was collected for further experiments and designated as ZJ06-200P5-1. MICs for colistin and tigecycline were determined by *E*-test (bioMérieux, France) on MH agar, and the antimicrobial activities of the other antimicrobial agents were detected by disk diffusion. The results were interpreted according to CLSI or EUCAST breakpoints.

Whole Genome DNA Sequencing and Analysis

ZJ06-200P5-1 cells were cultured from a single colony overnight at 37 °C in MH broth. The genomic DNA was extracted via a QIAamp DNA minikit (Qiagen, Valencia, CA) following the manufacturer's protocol. Agarose gel and a NanoDrop spectrophotometer were used to determine the quality and quantity of extracted genomic DNA. The 300 bp library for Illumina paired-end sequencing was constructed from 5 μg of genome DNA of ZJ06-200P5-1 by staff at Zhejiang Tianke (Hangzhou, China). Mapping and SNP detection were performed via Breseq (Deatherage and Barrick, 2014). The regions containing the detected SNPs were amplified by PCR. The PCR products were sent to Biosune (Biosune, Hangzhou, China) for Sanger sequencing.

TABLE 1 | Antibiotic susceptibility of *A. baumannii* MDR-ZJ06 and its colistin resistant mutant ZJ06-200P5-1.

Strains	CO[a]	TGC[a]	IPM	MEM	FEP	CAZ	CTX	ATM	PRL	TZP	SCF	SAM	CN	AK	TE	MH	CIP	CT
MDR-ZJ06	0.38 mg/L	4 mg/L	8	8	6	6	6	6	6	6	16	10	6	6	6	10	6	14
ZJ06-200P5-1	>256 mg/L	0.5 mg/L	22	22	20	20	15	22	17	19	30	22	6	6	8	26	9	6

CO, colistin; TGC, tigecycline; IPM, imipenem; MEM, meropenem; FEP, cefepime; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; PRL, Piperacillin; TZP, piperacillin/tazobactam; SCF, Cefoperazone/sulbactam; SAM, ampicillin/sulbactam; CN, gentamicin; AK, amikacin; TE, tetracycline; MH, minocycline; CIP, Ciprofloxacin; CT, colistin.

[a] *The MIC of colistin and tigecycline were determined by broth dilution method, while antimicrobial sensitivity of other antibiotics were detected by disk diffusion.*

TABLE 2 | Genes changed significantly in transcriptome.

Synonym	Product	logFC	logCPM	*P*-value	FDR
ABZJ_00055	hypothetical protein	8.308068	13.717	1.26E-78	4.54E-76
ABZJ_00068	hypothetical protein	6.4468	9.203574	2.14E-67	4.61E-65
ABZJ_00037	hypothetical protein	4.368832	9.669037	3.48E-68	9.36E-66
ABZJ_00056	hypothetical protein	4.349519	12.2059	6.03E-65	1.08E-62
ABZJ_00332	hypothetical protein	4.264896	9.455077	2.39E-53	2.86E-51
ABZJ_00036	hypothetical protein	3.449637	9.968726	9.61E-27	5.17E-25
ABZJ_01879	hypothetical protein	2.810666	6.769621	9.95E-35	7.65E-33
ABZJ_01880	putative transposase	2.758133	6.676606	5.52E-27	3.13E-25
ABZJ_01079	hypothetical protein	2.585295	6.001793	4.14E-10	6.55E-09
ABZJ_03753	hypothetical protein	2.318997	9.492231	2.51E-21	1.08E-19
ABZJ_00333	hypothetical protein	2.314205	5.437541	2.36E-11	4.53E-10
ABZJ_01881	transposase component	2.25458	8.338274	9.50E-21	3.93E-19
ABZJ_01133	heat shock protein	2.180889	13.35847	1.03E-25	5.06E-24
ABZJ_01180	putative phage-like protein	2.066152	3.22126	4.47E-06	3.56E-05
ABZJ_03752	PGAP1-like protein	2.014551	10.16569	2.49E-27	1.49E-25
ABZJ_00060	Thiol-disulfide isomerase and thioredoxin	1.894318	12.3252	7.68E-20	2.75E-18
ABZJ_00894	lactoylglutathione lyase-like protein	1.797874	6.779815	5.27E-15	1.62E-13
ABZJ_00054	N-alpha-acetylglutamate synthase (amino-acid acetyltransferase)	1.77044	10.25589	3.24E-20	1.27E-18
ABZJ_01151	hypothetical protein	1.634908	3.574211	4.88E-06	3.84E-05
ABZJ_03714	hypothetical protein	1.61859	8.500912	1.39E-08	1.85E-07
ABZJ_01900	acetoin:2,6-dichlorophenolindophenol oxidoreductase subunit alpha	1.527437	6.102611	2.98E-06	2.49E-05
ABZJ_01222	hypothetical protein	1.515854	2.111384	0.011897	0.034227
ABZJ_01191	hypothetical protein	1.46809	2.203352	0.011349	0.032877
ABZJ_01872	hypothetical protein	1.423713	7.613403	1.64E-08	2.10E-07
ABZJ_01187	hypothetical protein	1.423595	5.112417	2.82E-07	2.81E-06
ABZJ_01857	hypothetical protein	1.411761	2.566001	0.010144	0.029905
ABZJ_01829	Acyl-CoA dehydrogenase	1.402255	6.594396	4.45E-06	3.56E-05
ABZJ_01150	hypothetical protein	1.321675	3.205499	0.000936	0.003799
ABZJ_00028	lytic murein transglycosylase family protein	1.296752	10.96489	3.46E-14	9.79E-13
ABZJ_00976	hypothetical protein	1.295503	5.552053	1.46E-07	1.57E-06
ABZJ_01855	hypothetical protein	1.290522	2.587494	0.016132	0.044395
ABZJ_01186	hypothetical protein	1.249298	2.481015	0.013475	0.038054
ABZJ_00978	hypothetical protein	1.216859	3.038132	0.00684	0.021395
ABZJ_00977	hypothetical protein	1.209422	3.887522	0.000232	0.001118
ABZJ_00102	D-lactate dehydrogenase FAD-binding protein	1.170013	8.813908	1.91E-10	3.15E-09
ABZJ_01149	hypothetical protein	1.156232	3.314522	0.003302	0.011138
ABZJ_00053	alkanesulfonate transport protein	1.143156	6.421362	5.15E-06	3.99E-05
ABZJ_01275	hypothetical protein	1.122845	8.385252	1.31E-08	1.76E-07
ABZJ_03838	membrane-fusion protein	1.119324	7.708838	1.84E-08	2.33E-07
ABZJ_01901	acetoin:26-dichlorophenolindophenol oxidoreductase beta subunit	1.105826	6.349341	5.58E-05	0.000323
ABZJ_01899	lipoate synthase	1.08338	4.583472	0.003397	0.011422
ABZJ_00360	hypothetical protein	1.076106	8.065171	1.34E-07	1.46E-06
ABZJ_01210	hypothetical protein	1.065917	3.456549	0.011028	0.032156
ABZJ_01160	hypothetical protein	1.048988	3.144467	0.012194	0.034895
ABZJ_01148	hypothetical protein	1.048966	5.540519	1.77E-05	0.000122
ABZJ_00099	L-lactate permease	1.044891	10.0835	8.49E-08	9.61E-07
ABZJ_00901	major facilitator superfamily multidrug resistance protein	1.016944	9.235389	1.47E-08	1.91E-07
ABZJ_01775	6-pyruvoyl-tetrahydropterin synthase	1.014549	10.17374	3.05E-12	6.84E-11
ABZJ_03786	VirP protein	−1.0004	6.133241	3.35E-06	2.73E-05
ABZJ_01269	TPR repeat-containing SEL1 subfamily protein	−1.00222	4.702232	0.000305	0.001408

(Continued)

TABLE 2 | Continued

Synonym	Product	logFC	logCPM	*P*-value	FDR
ABZJ_00120	hypothetical protein	−1.00591	7.042084	6.25E-07	5.85E-06
ABZJ_00896	nucleoside-diphosphate sugar epimerase	−1.0079	7.57903	9.80E-07	8.86E-06
ABZJ_01258	hypothetical protein	−1.01127	4.48134	0.002855	0.009692
ABZJ_01260	metal ion ABC transporter substrate-binding protein/surface antigen	−1.01249	9.488595	2.29E-08	2.86E-07
ABZJ_01120	urease accessory protein UreE	−1.01439	6.914944	6.34E-07	5.88E-06
ABZJ_01873	hypothetical protein	−1.01999	5.846082	1.89E-05	0.000128
ABZJ_03812	hypothetical protein	−1.02082	4.567471	0.001409	0.005227
ABZJ_01101	hypothetical protein	−1.03046	5.533349	0.001752	0.006282
ABZJ_01908	Zn-dependent hydrolase, including glyoxylase	−1.03588	9.460654	2.53E-10	4.12E-09
ABZJ_03819	hypothetical protein	−1.05745	9.905586	6.08E-11	1.11E-09
ABZJ_03796	putative acyltransferase	−1.06273	6.680253	2.34E-07	2.42E-06
ABZJ_00947	hypothetical protein	−1.0641	6.738813	1.36E-06	1.21E-05
ABZJ_01169	hypothetical protein	−1.06442	8.404764	8.75E-07	7.98E-06
ABZJ_00345	hypothetical protein	−1.06443	6.560939	2.47E-07	2.53E-06
ABZJ_03828	hypothetical protein	−1.06567	4.05012	0.000406	0.001813
ABZJ_00922	hypothetical protein	−1.07121	5.599955	7.64E-05	0.000424
ABZJ_01907	response regulator	−1.07682	6.813752	2.94E-07	2.90E-06
ABZJ_03790	gamma-aminobutyrate permease	−1.07931	8.18838	3.71E-05	0.000227
ABZJ_00882	hypothetical protein	−1.07943	9.751157	2.22E-11	4.34E-10
ABZJ_01078	hypothetical protein	−1.08109	10.14275	5.68E-14	1.49E-12
ABZJ_01132	glutamate dehydrogenase/leucine dehydrogenase	−1.08366	7.760303	2.14E-07	2.24E-06
ABZJ_03802	putative homogentisate 1,2-dioxygenase	−1.08726	6.643847	0.000162	0.000822
ABZJ_00334	hypothetical protein	−1.09533	6.571739	7.17E-08	8.25E-07
ABZJ_01250	outer membrane receptor protein	−1.10965	7.442322	0.000193	0.000956
ABZJ_00367	hypothetical protein	−1.11395	8.476819	9.04E-09	1.25E-07
ABZJ_00946	hypothetical protein	−1.12668	5.862006	7.32E-06	5.59E-05
ABZJ_01265	hypothetical protein	−1.12706	10.47521	4.03E-13	9.42E-12
ABZJ_01257	Zn-dependent protease with chaperone function	−1.13229	6.680195	1.30E-05	9.11E-05
ABZJ_01110	putative hemolysin-related protein	−1.13995	9.22038	1.74E-11	3.54E-10
ABZJ_03720	UDP-3-O-acyl-N-acetylglucosamine deacetylase	−1.14429	8.585685	1.05E-05	7.52E-05
ABZJ_01960	isochorismate hydrolase	−1.14761	5.633402	0.000121	0.000638
ABZJ_00942	hypothetical protein	−1.15912	8.72549	8.38E-09	1.17E-07
ABZJ_03859	putative RND type efflux pump involved in aminoglycoside resistance (AdeT)	−1.17363	8.75427	3.19E-05	0.000202
ABZJ_01874	hypothetical protein	−1.17434	5.206346	2.41E-05	0.000159
ABZJ_01917	putative acyl carrier protein phosphodiesterase (ACP phosphodiesterase)	−1.18991	7.045816	5.55E-08	6.50E-07
ABZJ_01861	membrane-fusion protein	−1.20577	6.002924	1.77E-07	1.87E-06
ABZJ_03742	hypothetical protein	−1.20817	3.772045	0.001579	0.005748
ABZJ_01262	hypothetical protein	−1.21556	4.167491	8.53E-05	0.000466
ABZJ_01929	Aspartate ammonia-lyase (Aspartase)	−1.21837	11.63816	9.24E-14	2.31E-12
ABZJ_00924	hypothetical protein	−1.2423	8.464578	1.01E-10	1.79E-09
ABZJ_01155	hypothetical protein	−1.2668	10.80427	1.20E-16	3.79E-15
ABZJ_00388	2-polyprenyl-6-methoxyphenol hydroxylase	−1.26695	7.901741	1.19E-09	1.81E-08
ABZJ_01862	multidrug ABC transporter ATPase	−1.27715	6.94382	4.78E-09	6.86E-08
ABZJ_00944	hypothetical protein	−1.28276	5.658916	2.33E-08	2.88E-07
ABZJ_01156	hypothetical protein	−1.28415	8.332979	5.92E-11	1.10E-09
ABZJ_01826	AraC-type DNA-binding domain-containing protein	−1.29289	5.11387	5.57E-07	5.26E-06
ABZJ_03744	hypothetical protein	−1.29678	8.720807	1.08E-08	1.47E-07
ABZJ_03737	hypothetical protein	−1.30269	10.28829	3.31E-20	1.27E-18
ABZJ_00940	hypothetical protein	−1.30722	6.280622	2.75E-07	2.79E-06
ABZJ_01218	hypothetical protein	−1.30837	4.257169	9.06E-06	6.63E-05
ABZJ_00061	putative transcriptional regulator	−1.31564	7.634498	1.67E-10	2.80E-09

(Continued)

TABLE 2 | Continued

Synonym	Product	logFC	logCPM	*P*-value	FDR
ABZJ_01887	hypothetical protein	−1.3281	6.449578	1.02E-07	1.14E-06
ABZJ_01025	homocysteine/selenocysteine methylase	−1.33719	7.528478	3.07E-10	4.93E-09
ABZJ_00110	GNAT family acetyltransferase	−1.33942	4.887691	1.06E-06	9.50E-06
ABZJ_01242	hypothetical protein	−1.3506	7.369014	2.45E-09	3.61E-08
ABZJ_00895	hypothetical protein	−1.35351	6.693904	7.37E-12	1.56E-10
ABZJ_03712	putative flavoprotein	−1.38598	6.6067	2.04E-09	3.04E-08
ABZJ_00048	transcriptional regulator	−1.40027	7.755295	9.36E-11	1.68E-09
ABZJ_03785	glutamate racemase	−1.40496	7.417511	7.08E-12	1.52E-10
ABZJ_00938	hypothetical protein	−1.40799	6.629998	1.09E-10	1.88E-09
ABZJ_01230	hypothetical protein	−1.41279	10.19585	3.47E-19	1.20E-17
ABZJ_00124	glycine/D-amino acid oxidase (deaminating)	−1.46015	13.3987	8.58E-14	2.20E-12
ABZJ_03791	histidine ammonia-lyase (Histidase)	−1.49736	9.748038	2.37E-08	2.90E-07
ABZJ_03739	hypothetical protein	−1.49749	13.98113	3.54E-13	8.47E-12
ABZJ_00881	glutamine amidotransferase	−1.51327	8.144142	5.09E-14	1.37E-12
ABZJ_00988	hypothetical protein	−1.54819	6.1324	7.44E-09	1.05E-07
ABZJ_01840	putative ferric siderophore receptor protein	−1.55785	9.806018	9.74E-10	1.52E-08
ABZJ_00997	hypothetical protein	−1.58106	5.257799	3.12E-08	3.77E-07
ABZJ_00339	HSP90 family molecular chaperone	−1.6168	11.15864	7.57E-23	3.54E-21
ABZJ_00373	Type II secretory pathway, ATPase PulE/Tfp pilus assembly pathway, ATPase PilB	−1.6419	6.706339	3.45E-14	9.79E-13
ABZJ_01845	phosphatase/phosphohexomutase	−1.68301	7.222507	3.67E-12	8.06E-11
ABZJ_03793	urocanate hydratase	−1.69267	10.89217	1.13E-07	1.25E-06
ABZJ_03754	Rhs element Vgr family protein	−1.69503	8.757228	5.86E-18	1.97E-16
ABZJ_00945	hypothetical protein	−1.72533	5.192791	2.02E-11	4.03E-10
ABZJ_01002	putative ABC oligo/dipeptide transport, ATP-binding protein	−1.73182	6.449009	4.32E-14	1.19E-12
ABZJ_01259	hypothetical protein	−1.75565	7.198513	1.30E-12	2.98E-11
ABZJ_00114	short chain dehydrogenase family protein	−1.76754	7.176594	1.03E-13	2.52E-12
ABZJ_01177	hypothetical protein	−1.8053	8.135954	6.06E-15	1.81E-13
ABZJ_03792	hypothetical protein	−1.82418	6.284478	3.56E-06	2.88E-05
ABZJ_01219	hypothetical protein	−1.86448	9.22858	7.68E-22	3.45E-20
ABZJ_01088	carbonic anhydrase	−1.94984	9.430551	1.08E-27	6.83E-26
ABZJ_00346	hypothetical protein	−2.03948	6.219886	1.15E-16	3.73E-15
ABZJ_01207	hypothetical protein	−2.1746	7.126199	6.11E-20	2.27E-18
ABZJ_01886	hypothetical protein	−2.33548	5.458495	1.05E-11	2.18E-10
ABZJ_03766	putative secretory lipase precursor	−2.38284	9.073946	1.11E-31	7.47E-30
ABZJ_01206	hypothetical protein	−3.28101	9.194837	2.48E-45	2.42E-43
ABZJ_03736	thiol:disulfide interchange protein	−3.9361	9.872762	6.64E-41	5.50E-39

Transcriptome Analysis and Real-Time Quantitative PCR Verification

A. baumannii MDR-ZJ06 and ZJ06-200P5-1 were grown overnight at 37 °C in LB broth. Strains were subcultured 1/100 into fresh LB broth and grown at 37 °C for 2 h (OD_{600}: 0.29 ± 0.02 for MDR-ZJ06, 0.26 ± 0.02 for ZJ06-200P5-1). The cells were collected at 4 °C, and the RNA was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) after liquid nitrogen grinding. For RNA sequencing, wild type and mutants were sampled in triplicate. The subsequent RNA extraction, bacteria mRNA sequence library construction, transcriptome analysis and real-time quantitative PCR verification were performed by staff at Zhejiang Tianke (Hangzhou, China) as described previously in reference (Hua et al., 2014). Sequenced reads were mapped to the MDR-ZJ06 genome (CP001937-8) using Rockhopper (McClure et al., 2013). The output data was analyzed by edgeR (McCarthy et al., 2012). Data generated by RNA sequencing were deposited to the NCBI Sequence Read Archive with accession number SRR5234544 (the wild type) and SRR5234545 (the colistin resistant strain).

Proteomic Analysis

A. baumannii MDR-ZJ06 and ZJ06-200P5-1 were grown overnight at 37 °C in LB broth. Strains were subcultured 1/100 into fresh LB broth and grown at 37 °C for 2 h (OD_{600}: 0.29 ± 0.02 for MDR-ZJ06, 0.26 ± 0.02 for ZJ06-200P5-1). The cells were collected at 4 °C and sent to Shanghai Applied Protein Technology Co. Ltd. The cell pellets were washed twice with PBS, and 500 μl SDT lysis buffer (4% SDS, 100 mM Tris-HCl, 1 mM DTT, pH 7.6) was added. After being sonicated for 2 mins on ice,

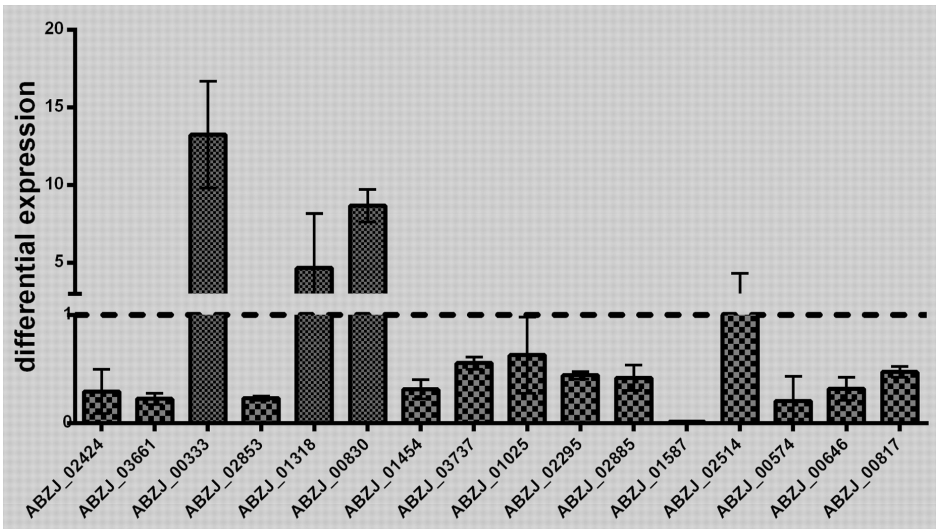


FIGURE 2 | Validation of the RNA sequencing results. The transcriptomic results obtained by RNA-seq were validated by quantitative RT-PCR analysis. The differential expression of 16 genes was detected in this study. Three biology replicates were used in this experiment. The results were presented as expression in ZJ06-200P5-1, relative to MDR-ZJ06. The reference gene *rpoB* was used for inter-sample normalization. Error bars denote standard deviation.

the cells were centrifuged at 14,000 × g for 30 min at 4 °C. The protein concentration in the supernatant was determined by the BCA method.

In brief, 300 μg protein was added to 200 μl UA buffer (8 M urea, 150 mM Tris-HCl pH 8.0) and ultrafiltered (Sartorius, 10 kD) with UA buffer. To block reduced cysteine residues, 100 μl iodoacetamide (IAA) buffer (50 mM IAA in UA buffer) was added, centrifuged at 600 rpm for 1 min, and incubated for 30 min in the dark. The filter was washed twice with 100 μl UA buffer and twice with 100 μl Dissolution buffer (50 mM triethylammonium bicarbonate, pH 8.5). Finally, the proteins were digested with 2 μg trypsin (Promega) in 40 μl Dissolution buffer at 37 °C for 16–18 h. The peptides were collected as a filtrate, and its content was estimated at OD_{280}.

For iTRAQ labeling, the peptides were labeled with the 4-plex iTRAQ reagent following the manufacturer's instructions (AB SCIEX). The peptides from MDR-ZJ06 were labeled with 114 and 116 isobaric reagents, and the peptides from ZJ06-200P5-1 were labeled with 115 and 117 isobaric reagents.

RP-HPCL online-coupled to MS/MS (LC-MS/MS) analysis of the iTRAQ-labeled peptides was performed on an EASY-nLC nanoflow LC system (Thermo Fisher Scientific) connected to an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific). After the samples were reconstituted and acidified with buffer A (0.1% (v/v) formic acid in water), a set-up involving a pre-column and analytical column was used. The pre-column was a 2 cm EASY-column (100, 5 μm C18; Thermo Fisher Scientific), while the analytical column was a 10 cm EASY-column (75, 3 μm, C18; Thermo Fisher Scientific). The 120 min linear gradient from 0 to 100% buffer B (0.1% (v/v) formic acid and 80% acetonitrile) at a constant flow rate of 250 nl/min was as follows: 0–100 min, 0–35% buffer B; 100–108 min, 35–100% buffer B; 108–120 min, 100% buffer B. MS data were acquired using a data-dependent top 10 method, dynamically choosing the most abundant precursor ions from the survey scan (300–180 m/z) for HCD fragmentation. The Dynamic exclusion was set to a repeat count of 1 with a 30 s duration. Survey scans were acquired at a resolution of 30,000 at m/z 200, and the resolution for HCD spectra was set to 15,000 at m/z 200. The normalized collision energy was 35 eV, and the underfill ratio was defined as 0.1%.

The MS/MS spectra were searched using the MASCOT engine (Matrix Science, London, UK; version 2.2) against the *A. baumannii* MDR-ZJ06 FASTA database. False discovery rates (FDR) were calculated via running all spectra against the FASTA database using the MASCOT software. The following options were used to identify proteins: peptide mass tolerance = 20 ppm, fragment mass tolerance = 0.1 Da, Enzyme = Trypsin, Max missed cleavages = 2, Fixed modification: Carbamidomethyl (C), iTRAQ 4plex (N-term), iTRAQ 4plex (K), Variable modification: Oxidation (M). Quantification was performed based on the peak intensities of the reporter ions in the MS/MS spectra. The proteins were considered overexpressed when the iTRAQ ratio was above 1.5 and underexpressed when the iTRAQ ratio was lower than 0.67 (Wang et al., 2016). Functional classification of differentially expression genes were annotated using the KEGG databases. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD005265 and 10.6019/PXD005265. Reviewer account details: Username: reviewer54242@ebi.ac.uk; Password: zR8mE9wu.

Growth Rate Determination

Four independent cultures per strain were grown overnight, diluted to 1:1000 in MH and aliquots placed into a flat-bottom 100-well plate in four replicates. The plate was incubated at 37 °C

with agitation. The OD_{600} of each culture was determined every 5 min for 16 h using a Bioscreen C MBR machine (Oy Growth Curves Ab Ltd., Finland). The growth rate was estimated based on OD_{600} curves using an R script (Fang et al., 2016).

RESULTS

Whole Genome Sequencing, Minimum Inhibitory Concentration and Growth Rate

The colistin-resistant mutant ZJ06-200P5-1 generated from the culture in CAMHB containing colistin was sent for whole genome sequencing. There was an ISAba1 insertion in *lpxC* in ZJ06-200P5-1 compared with the genome sequence of MDR-ZJ06 (**Figure 1**). The MIC of MDR-ZJ06 and ZJ06-200P5-1 were detected and listed in **Table 1**. The MIC for colistin increased from 0.38 mg/L (MDR-ZJ06) to >256 mg/L (ZJ06-200P5-1). However, ZJ06-200P5-1 showed higher sensitivity to multiple antibiotics: β-lactams, carbapenem, tetracycline, and ciprofloxacin, but not aminoglycosides. Furthermore, ZJ06-200P5-1 showed a lower growth rate (0.81 ± 0.05) than wild type.

Transcriptome Analysis

The transcriptome analysis of ZJ06-200P5-1 and MDR-ZJ06 was performed by Illumina RNA deep sequencing technology. Cells of the two strains were collected in the early exponential phase. A total of 137 genes showed significant differential expression [log2(FoldChange) $>$ 1 or log2(FoldChange) $<$ -1], among which 48 genes were upregulated and 89 were downregulated (**Table 2**). Sixteen selected genes, three up-regulated and thirteen down-regulated genes, were well-validated by RT-qPCR (**Figure 2**). After mapping the differentially expressed genes into the KEGG pathway, we observed that genes involved in Energy metabolism and Amino acid metabolism were down-regulated, while Carbohydrate metabolism was up-regulated.

iTRAQ

A total of 1582 proteins were identified in the iTRAQ experiment. A protein ratio >1.5 or <0.67 ($p < 0.05$) was considered to be differentially expressed. After filtration, 82 differentially expressed proteins were identified between ZJ06-200P5-1 and MDR-ZJ06. The detailed information is shown in **Table 3**.

The expression of AdeABC was up-regulated in the LPS-loss ZJ06-200P5-1 strain. The AdeABC efflux pump confers resistance to various antibiotics classes. The expression of AdeABC genes was increased approximately two-fold in ZJ06-200P5-1 (**Figure 3A**). However, ZJ06-200P5-1 showed higher susceptibility to multiple antibiotics than MDR-ZJ06 (**Table 1**).

The fatty acid biosynthesis pathway was down-regulated in the ZJ06-200P5-1 strain (**Figure 3B**). The expression of FabZ was decreased by approximately two-fold in ZJ06-200P5-1. The β-lactamases bla_{OXA-23} and bla_{ADC-25} were down-regulated in ZJ06-200P5-1 strain. The expression levels of bla_{OXA-23} and bla_{ADC-25} were decreased two- to four-fold in ZJ06-200P5-1.

Common Genes Altered Expression in Both Transcriptome and Proteome

A total of 15 differentially expressed genes (or proteins) were identified in both transcriptome and proteome (**Table 4**). Among them, three genes were both up-regulated, and nine genes were both down-regulated. Although there was correlation between transcriptome and proteome data, the absolute expression difference values in transcriptome data was higher than those in proteome data. In addition, the result of three gene/proteins were contradictory (highlighted in red letters in **Table 4**). The contradictory result might be caused by post-transcriptional regulation.

DISCUSSION

Due to the limitation of antimicrobial agents in clinical use, it is urgent to extend our understanding of the emergence of colistin resistance in *A. baumannii*. *A. baumannii* MDR-ZJ06, a multidrug-resistant clinical strain isolated from bloodstream, has been sequenced and was considered an ideal strain for examining the colistin-resistant mechanism in *A. baumannii* (Zhou et al., 2011). In this study, colistin-resistant strain was rapidly obtained, and its resistance mechanism was LPS loss caused by IS*Aba1* insertion in *lpxC*. This result confirmed a previous finding (Moffatt et al., 2010). The rapid isolation of colistin-resistant mutant from multiple drug-resistant *A. baumannii* indicated a high risk of *A. baumannii* evolving resistance to colistin in clinical use.

We successfully detected the whole transcriptional profile of *A. baumannii* strain MDR-ZJ06 and its colistin-resistant mutant ZJ06-200P5-1 via Illumina RNA-sequencing. In another transcriptome study (Henry et al., 2012), *A. baumannii* ATCC 19606 and its *lpxA* mutant were used. Although both the *lpxC* and *lpxA* mutation lead to LPS loss, the different transcriptional response may be due to differences in the strain genetic background and the resistant mutation. In transcriptional analysis, we observed that genes involved in Energy metabolism and Amino acid metabolism were down-regulated, while Carbohydrate metabolism was up-regulated.

The expression of AdeABC was up-regulated in the LPS-loss ZJ06-200P5-1 strain. Similar results were also observed in all polymyxin-treated samples (Cheah et al., 2016a). In addition, the expression levels of *adeIJK* and *macAB-tolC* were up-regulated in the LPS loss mutant (Henry et al., 2012). Increased expression of the RND efflux pump system (AdeABC) was a common finding across all experiments in colistin exposure. The up-regulation of AdeABC indicated the diminished integrity and barrier function of the outer membrane in colistin-resistant *A. baumannii* (Henry et al., 2015; Cheah et al., 2016a). However, ZJ06-200P5-1 showed higher susceptibility to multiple antibiotics than MDR-ZJ06. The higher susceptibility might result from the higher outer membrane permeability of ZJ06-200P5-1 due

TABLE 3 | Genes changed significantly in proteome.

Protein number	NCBInr acession	Gene tag	Protein description	Pep Count	Unique PepCount	Coverage (%)	MW	pI	log2 of ratio (ZJ06-200P5-1 vs. MDR-ZJ06)	*p*-value
233	384144952	ABZJ_03706	hypothetical protein	75	12	66.27	27649.89	4.59	1.65184	2.90E-20
1280	384143756	ABZJ_02510	hypothetical protein	1	1	10.18	17235.79	10.09	1.49121	8.79E-17
756	384144562	ABZJ_03316	hypothetical protein	27	4	34.13	13935.85	9.67	1.49075	8.99E-17
1032	384144568	ABZJ_03322	hypothetical protein	7	2	15.75	15550.26	10.03	1.39649	6.82E-15
565	384143898	ABZJ_02652	hypothetical protein	23	6	54.76	13282.22	8.99	1.15312	1.36E-10
594	384141430	ABZJ_00184	hypothetical protein	14	6	32.66	22273.87	4.56	1.131	3.05E-10
1241	384141854	ABZJ_00608	dehydrogenase	1	1	5.13	30137.1	8.79	1.11427	5.57E-10
1188	384141579	ABZJ_00333	hypothetical protein	5	1	10.66	11110.55	9.66	1.09309	1.18E-09
1076	384143755	ABZJ_02509	hypothetical protein	4	2	31.91	13701.31	10.29	1.09014	1.31E-09
147	384141823	ABZJ_00577	membrane-fusion protein	59	17	45.29	48231.1	9.44	0.9855	4.28E-08
1209	384142731	ABZJ_01485	dihydrodipicolinate synthase	2	1	2.89	33837.12	5.46	0.956837	1.05E-07
175	384143251	ABZJ_02005	membrane-fusion protein	50	15	47.22	43375.8	7.75	0.9115	4.13E-07
1281	384143760	ABZJ_02514	glycosyltransferase	1	1	3.37	48412.32	9.23	0.889123	7.92E-07
454	384141821	ABZJ_00575	putative outer membrane protein	18	8	21.57	54556.06	8.52	0.886277	8.60E-07
1009	384141578	ABZJ_00332	hypothetical protein	26	2	32.23	11005.53	9.93	0.859413	1.84E-06
1216	384143670	ABZJ_02424	hypothetical protein	2	1	25.58	4520.08	5.45	0.848157	2.51E-06
201	384143250	ABZJ_02004	cation/multidrug efflux pump	26	14	15.64	112744.8	7.6	0.801366	8.82E-06
885	384142076	ABZJ_00830	Outer membrane lipoprotein	12	3	18.75	21087.72	6.9	0.801241	8.85E-06
323	384144243	ABZJ_02997	putative porin protein associated with imipenem resistance	97	10	50.81	26505.22	4.8	0.770322	1.96E-05
1029	384141822	ABZJ_00576	peptide ABC transporter permease	7	2	3.77	71261.81	6.24	0.753391	2.98E-05
164	384144912	ABZJ_03666	NAD-dependent aldehyde dehydrogenase	41	16	43.15	51846.55	5.11	0.751721	3.11E-05
655	384144155	ABZJ_02909	hypothetical protein	27	5	33.48	26172.15	7.85	0.733875	4.80E-05
812	384142146	ABZJ_00900	multidrug resistance secretion protein	8	4	9.14	40956.99	6.56	0.691132	0.000131
852	384145008	ABZJ_03762	putative short-chain dehydrogenase	6	4	17.24	31854.29	9.26	0.688359	0.000139
539	384144680	ABZJ_03434	flavoprotein	10	7	15.52	55720.24	9.12	0.685088	0.00015
150	384144913	ABZJ_03667	4-aminobutyrate aminotransferase	55	17	50.23	45976.96	5.81	0.679784	0.000169
1306	384144561	ABZJ_03315	kinase sensor component of a two component signal transduction system	1	1	3.07	62690.76	6.3	0.672652	0.000198
600	384144948	ABZJ_03702	xenobiotic reductase	14	6	21.02	38725.16	5.08	0.608194	0.000783

(Continued)

TABLE 3 | Continued

Protein number	NCBInr acession	Gene tag	Protein description	Pep Count	Unique PepCount	Coverage (%)	MW	pI	log2 of ratio (ZJ06-200P5-1 vs. MDR-ZJ06)	*p*-value
315	384144930	ABZJ_03684	hypothetical protein	322	10	47.37	32732.07	4.71	0.602647	0.000876
603	384143541	ABZJ_02295	UDP-glucose 4-epimerase	13	6	28.06	38064.02	5.53	0.599175	0.000939
384	384142564	ABZJ_01318	Zn-dependent protease with chaperone function	35	9	48.66	27572.18	9.44	0.592971	0.001063
680	384143417	ABZJ_02171	hypothetical protein	14	5	40.65	17046.41	8.79	−0.59205	0.000855
996	384143586	ABZJ_02340	hypothetical protein	3	3	10.61	29941.63	6.85	−0.60757	0.000626
1007	384145105	ABZJ_03859	putative RND type efflux pump involved in aminoglycoside resistance (AdeT)	3	3	10.48	38641.56	9.71	−0.60779	0.000623
667	384144990	ABZJ_03744	hypothetical protein	18	5	21.99	27747.62	4.62	−0.60878	0.00061
820	384141318	ABZJ_00072	FKBP-type 22KD peptidyl-prolyl cis-trans isomerase	7	4	21.65	25217.38	9.06	−0.61264	0.000564
767	384141553	ABZJ_00307	hypothetical protein	17	4	48.31	10746.92	5.3	−0.61297	0.00056
163	384144907	ABZJ_03661	hypothetical protein	47	16	39.91	49757.27	8.16	−0.61374	0.000551
865	384144338	ABZJ_03092	Zn-dependent hydrolase, including glyoxylase	5	4	15.00	35333.86	8.91	−0.62839	0.000407
780	384141775	ABZJ_00529	gluconate kinase	12	4	30.59	18924.48	4.88	−0.6352	0.000353
1259	384142716	ABZJ_01470	hypothetical protein	1	1	2.52	36304.38	9.04	−0.63588	0.000348
424	384142064	ABZJ_00818	3-oxoacyl-ACP reductase	42	8	45.90	26098.39	6.1	−0.64296	0.000299
825	384141812	ABZJ_00566	hypothetical protein	7	4	36.11	15329.44	9.46	−0.64431	0.00029
381	384141306	ABZJ_00060	Thiol-disulfide isomerase and thioredoxin	37	9	42.44	22825.09	9.58	−0.65529	0.000229
963	384142833	ABZJ_01587	dehydrogenase	4	3	9.93	31970.72	5.16	−0.6827	0.000125
645	384141583	ABZJ_00337	putative outer membrane protein W	52	5	28.64	22680.64	5.9	−0.69549	9.35E-05
329	384142063	ABZJ_00817	malonyl-CoA-[acyl-carrier-protein] transacylase	59	10	43.15	35339.2	5.22	−0.6997	8.49E-05
941	384142271	ABZJ_01025	homocysteine/selenocysteine methylase	5	3	12.33	32062.1	4.82	−0.71762	5.59E-05
716	384144502	ABZJ_03256	protein-disulfide isomerase	9	5	23.31	26361.06	9	−0.72106	5.15E-05
232	384144545	ABZJ_03299	acetylCoA carboxylase subunit beta	76	12	44.63	32971.73	5.85	−0.72297	4.93E-05
836	384144135	ABZJ_02889	hypothetical protein	7	4	38.57	15413.52	8.43	−0.72309	4.91E-05
207	384141892	ABZJ_00646	Acetyl-CoA carboxylase alpha subunit	87	13	75.09	29640.53	5.6	−0.72798	4.37E-05
1053	384144131	ABZJ_02885	LysR family transcriptional regulator	5	2	6.80	34516.26	6.26	−0.74843	2.67E-05

(Continued)

TABLE 3 | Continued

Protein number	NCBInr acession	Gene tag	Protein description	Pep Count	Unique PepCount	Coverage (%)	MW	pI	log2 of ratio (ZJ06-200P5-1 vs. MDR-ZJ06)	*p*-value
883	384142465	ABZJ_01219	hypothetical protein	14	3	26.54	17636.93	9.58	−0.75975	2.02E-05
791	384142700	ABZJ_01454	hypothetical protein	10	4	25.15	19116.5	5	−0.77251	1.47E-05
573	384144158	ABZJ_02912	putative fatty acid desaturase	20	6	17.03	42202.21	9.39	−0.77608	1.34E-05
663	384141673	ABZJ_00427	putative type III effector HopPmaJ	19	5	37.27	12074.21	5.41	−0.78501	1.07E-05
261	384141776	ABZJ_00530	NAD-dependent aldehyde dehydrogenase	28	12	22.69	60150.9	6.04	−0.80138	7.02E-06
166	384141820	ABZJ_00574	NADH-dependent enoyl-ACP reductase	142	15	64.24	31016.41	6	−0.81807	4.53E-06
280	384144728	ABZJ_03482	putative toluene tolerance protein (Ttg2D)	76	11	61.97	23513.33	9.83	−0.82764	3.51E-06
917	384142976	ABZJ_01730	hypothetical protein	7	3	14.80	21011.63	9.2	−0.8625	1.36E-06
192	384144009	ABZJ_02763	hypothetical protein	63	14	48.19	44493.93	8.79	−0.86539	1.25E-06
635	384144826	ABZJ_03580	putative penicillin binding protein (PonA)	8	6	8.23	94767.31	9.38	−0.88231	7.77E-07
292	384142962	ABZJ_01716	biotin synthetase	40	11	34.83	37136.95	5.45	−0.89634	5.20E-07
909	384142828	ABZJ_01582	putative 17 kDa surface antigen	8	3	44.76	12431.23	4.7	−0.93167	1.85E-07
483	384144247	ABZJ_03001	hypothetical protein	43	7	48.55	14704.84	9.54	−0.93498	1.67E-07
188	384142100	ABZJ_00854	beta-ketoacyl-ACP synthase	90	14	46.45	43130.17	5.2	−0.94675	1.17E-07
446	384144999	ABZJ_03753	hypothetical protein	22	8	39.09	28038.81	9.07	−0.95428	9.33E-08
401	384144159	ABZJ_02913	flavodoxin reductase (ferredoxin-NADPH reductase) family protein 1	23	9	31.46	39570.7	6.09	−0.95498	9.13E-08
489	384143515	ABZJ_02269	(3R)-hydroxymyristoyl-ACP dehydratase	39	7	50.93	17988.69	6.3	−0.97767	4.53E-08
459	384142835	ABZJ_01589	hypothetical protein	18	8	13.33	43721.35	4.96	−0.97866	4.39E-08
833	384143336	ABZJ_02090	hypothetical protein	7	4	37.91	17951.03	4.82	−1.00115	2.16E-08
586	384143810	ABZJ_02564	hypothetical protein	16	6	79.22	8718.62	5	−1.02063	1.15E-08
114	384143236	ABZJ_01990	beta-lactamase OXA-23	161	18	71.38	31385.05	8.37	−1.0965	8.98E-10
359	384143517	ABZJ_02271	putative outer membrane protein (OmpH)	25	10	57.49	18710.09	9.52	−1.22331	8.58E-12
606	384144983	ABZJ_03737	hypothetical protein	13	6	38.04	27580.52	4.68	−1.28419	7.75E-13
95	384144431	ABZJ_03185	putative DcaP-like protein	111	20	50.69	47278.17	6.37	−1.36068	3.23E-14
939	384141906	ABZJ_00660	putative lipoprotein precursor (VacJ) transmembrane	5	3	10.67	33499.98	4.85	−1.43792	1.09E-15
1289	384144099	ABZJ_02853	hypothetical protein	1	1	8.06	14811.21	4.39	−1.58122	1.26E-18
1186	384142065	ABZJ_00819	acyl carrier protein (ACP)	21	1	10.99	10132.23	4.11	−1.65109	3.70E-20
412	384142699	ABZJ_01453	hypothetical protein	14	9	46.52	25412.88	9.89	−1.66497	1.81E-20
113	384144004	ABZJ_02758	beta-lactamase	268	18	55.05	44683.92	9.28	−1.82062	3.88E-24

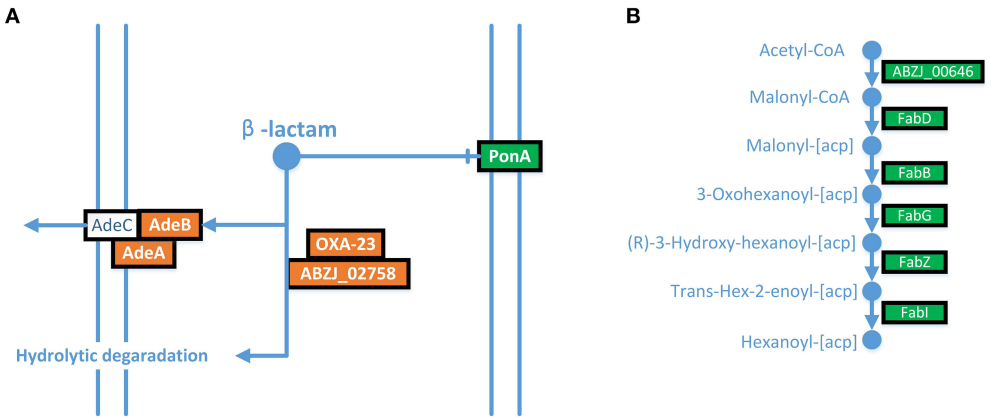


FIGURE 3 | ITRAQ analysis showed that AdeABC were up-regulated, and the fatty acid biosynthesis pathway was down-regulated in ZJ06-200P5-1. **(A)** AdeABC efflux pump, **(B)** fatty acid biosynthesis pathway. Green shows genes with significantly reduced expression levels, and red shows genes with significantly increased expression levels.

to LPS-loss. The increased expression of the efflux pump was thought to be a response to toxic substances that accumulated in the cells due to the increased membrane permeability (Henry et al., 2012).

The fatty acid biosynthesis pathway was down-regulated in the ZJ06-200P5-1 strain. In *E. coli*, it is important to balance LPS and fatty acid biosynthesis to maintain cell integrity. FabZ, which dehydrates R-3-hydroxymyristoyl-acyl carrier protein in fatty acid biosynthesis, plays an important role in rebalancing lipid A and fatty acid homeostasis (Bojkovic et al., 2016). The decrease in FabZ was considered to be a response to LPS-loss in ZJ06-200P5-1. The β-lactamases bla_{OXA-23} and bla_{ADC-25} were down-regulated in the ZJ06-200P5-1 strain. Decreased expression levels of bla_{OXA-23} and bla_{ADC-25} were also observed in *A. baumannii* MDR-ZJ06 under a subinhibitory concentration of tigecycline (Hua et al., 2014). Meanwhile, the strain under tigecycline stress showed a lower MIC of ceftazidime (Hua et al., 2014). The decrease in bla_{OXA-23} and bla_{ADC-25} might contribute to the increased sensitivity to β-lactam antimicrobial agents.

A multi-omics approach was adopted to obtain a more global view of colistin-resistant *A. baumannii*. Genomic analysis showed that *lpxC* was inactivated by IS*Aba1* insertion, leading to LPS loss. Transcriptional analysis demonstrated that the colistin-resistant strain regulated its metabolism. Metabolic change and LPS loss were concomitant. Proteomic analysis suggested increased expression of the RND efflux pump system and the down-regulation of FabZ and β-lactamase. These alterations are believed to be responses to LPS loss. Together, the *lpxC* mutation not only confirmed colistin resistance but also altered global gene expression.

Nucleotide Sequence Accession Numbers

The whole-genome shotgun sequencing results for *A. baumannii* ZJ06-200P5-1 have been deposited at DDBJ/EMBL/GenBank under the accession number MIFW00000000.

TABLE 4 | Common genes altered expression both in transcriptome and proteome.

Synonym	Product	Fold change (log2, Transcriptome)	Fold change (log2, Proteome)
ABZJ_00332	hypothetical protein	4.26489563	0.859413
ABZJ_03753	hypothetical protein	2.318997325[a]	−0.95428
ABZJ_00333	hypothetical protein	2.314204886	1.09309
ABZJ_01133	heat shock protein	2.180888936	0.532117
ABZJ_00060	Thiol-disulfide isomerase and thioredoxin	1.894317881[a]	−0.65529
ABZJ_00028	lytic murein transglycosylase family protein	1.296751692[a]	−0.57293
ABZJ_01078	hypothetical protein	−1.081092562	−0.44448
ABZJ_03720	UDP-3-O-acyl-N-acetylglucosamine deacetylase	−1.144287283	−0.48378
ABZJ_03859	putative RND type efflux pump involved in aminoglycoside resistance (AdeT)	−1.173634714	−0.60779
ABZJ_03744	hypothetical protein	−1.296782077	−0.60878
ABZJ_03737	hypothetical protein	−1.302692756	−1.28419
ABZJ_01025	homocysteine/ selenocysteine methylase	−1.337189269	−0.71762
ABZJ_01219	hypothetical protein	−1.864476303	−0.75975
ABZJ_01088	carbonic anhydrase	−1.949843631	−0.56001
ABZJ_01206	hypothetical protein	−3.281014801	−0.4346

[a] *The result of three gene/proteins were contradictory.*

AUTHOR CONTRIBUTIONS

XH and YY conceived and designed the study. XH, LL, YF, QS, XL, QC, KS, YJ, and HZ performed the experiments. XH and YY performed data analysis and drafted the manuscript. All authors reviewed and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (81230039, 31670135, 81378158), the 973 Preliminary Research Program (2014CB560707), the Natural Science Foundation of Zhejiang province, China (LY15H190004, Y16H190013) and the Zhejiang Province Medical Platform Backbone Talent Plan (2016DTA003).

REFERENCES

Antunes, L. C., Visca, P., and Towner, K. J. (2014). *Acinetobacter baumannii*: evolution of a global pathogen. *Pathog. Dis.* 71, 292–301. doi: 10.1111/2049-632X.12125

Bae, S., Kim, M. C., Park, S. J., Kim, H. S., Sung, H., Kim, M. N., et al. (2016). *In vitro* synergistic activity of antimicrobial agents in combination against clinical isolates of colistin-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother.* 60, 6774–6779. doi: 10.1128/AAC.00839-16

Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J. A., Doumith, M., Hornsey, M., et al. (2011). Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the pmrAB two-component regulatory system. *Antimicrob. Agents Chemother.* 55, 3370–3379. doi: 10.1128/AAC.00079-11

Beceiro, A., Moreno, A., Fernandez, N., Vallejo, J. A., Aranda, J., Adler, B., et al. (2014). Biological cost of different mechanisms of colistin resistance and their impact on virulence in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 58, 518–526. doi: 10.1128/AAC.01597-13

Bojkovic, J., Richie, D. L., Six, D. A., Rath, C. M., Sawyer, W. S., Hu, Q., et al. (2016). Characterization of an *Acinetobacter baumannii* lptD deletion strain: permeability defects and response to inhibition of lipopolysaccharide and fatty acid biosynthesis. *J. Bacteriol.* 198, 731–741. doi: 10.1128/JB.00639-15

Cai, Y., Chai, D., Wang, R., Liang, B., and Bai, N. (2012). Colistin resistance of *Acinetobacter baumannii*: clinical reports, mechanisms and antimicrobial strategies. *J. Antimicrob. Chemother.* 67, 1607–1615. doi: 10.1093/jac/dks084

Cheah, S. E., Johnson, M. D., Zhu, Y., Tsuji, B. T., Forrest, A., Bulitta, J. B., et al. (2016a). Polymyxin resistance in *Acinetobacter baumannii*: genetic mutations and transcriptomic changes in response to clinically relevant dosage regimens. *Sci. Rep.* 6:26233. doi: 10.1038/srep26233

Cheah, S. E., Li, J., Tsuji, B. T., Forrest, A., Bulitta, J. B., and Nation, R. L. (2016b). Colistin and polymyxin B dosage regimens against *Acinetobacter baumannii*: differences in activity and the emergence of resistance. *Antimicrob. Agents Chemother.* 60, 3921–3933. doi: 10.1128/AAC.02927-15

Deatherage, D. E., and Barrick, J. E. (2014). Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods Mol. Biol.* 1151, 165–188. doi: 10.1007/978-1-4939-0554-6_12

Fang, L., Chen, Q., Shi, K., Li, X., Shi, Q., He, F., et al. (2016). Step-Wise increase in tigecycline resistance in *klebsiella pneumoniae* associated with Mutations in ramR, lon and rpsJ. *PLoS ONE* 11:e0165019. doi: 10.1371/journal.pone.0165019

Fernandez-Reyes, M., Rodriguez-Falcon, M., Chiva, C., Pachon, J., Andreu, D., and Rivas, L. (2009). The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective. *Proteomics* 9, 1632–1645. doi: 10.1002/pmic.200800434

Henry, R., Crane, B., Powell, D., Deveson Lucas, D., Li, Z., Aranda, J., et al. (2015). The transcriptomic response of *Acinetobacter baumannii* to colistin and doripenem alone and in combination in an *in vitro* pharmacokinetics/pharmacodynamics model. *J. Antimicrob. Chemother.* 70, 1303–1313. doi: 10.1093/jac/dku536

Henry, R., Vithanage, N., Harrison, P., Seemann, T., Coutts, S., Moffatt, J. H., et al. (2012). Colistin-resistant, lipopolysaccharide-deficient *Acinetobacter baumannii* responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-beta-1,6-N-acetylglucosamine. *Antimicrob. Agents Chemother.* 56, 59–69. doi: 10.1128/AAC.05191-11

Howard, A., O'Donoghue, M., Feeney, A., and Sleator, R. D. (2012). *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence* 3, 243–250. doi: 10.4161/viru.19700

Hua, X., Chen, Q., Li, X., and Yu, Y. (2014). Global transcriptional response of *Acinetobacter baumannii* to a subinhibitory concentration of tigecycline. *Int. J. Antimicrob. Agents* 44, 337–344. doi: 10.1016/j.ijantimicag.2014.06.015

Li, J., Rayner, C. R., Nation, R. L., Owen, R. J., Spelman, D., Tan, K. E., et al. (2006). Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 50, 2946–2950. doi: 10.1128/AAC.00103-06

McCarthy, D. J., Chen, Y., and Smyth, G. K. (2012). Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* 40, 4288–4297. doi: 10.1093/nar/gks042

McClure, R., Balasubramanian, D., Sun, Y., Bobrovskyy, M., Sumby, P., Genco, C. A., et al. (2013). Computational analysis of bacterial RNA-Seq data. *Nucleic Acids Res.* 41:e140. doi: 10.1093/nar/gkt444

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D., Vinogradov, E., Seemann, T., et al. (2010). Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob. Agents Chemother.* 54, 4971–4977. doi: 10.1128/AAC.00834-10

Mu, X., Wang, N., Li, X., Shi, K., Zhou, Z., Yu, Y., et al. (2016). The Effect of Colistin Resistance-Associated Mutations on the Fitness of *Acinetobacter baumannii*. *Front. Microbiol.* 7:1715. doi: 10.3389/fmicb.2016.01715

Peleg, A. Y., Seifert, H., and Paterson, D. L. (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbiol. Rev.* 21, 538–582. doi: 10.1128/CMR.00058-07

Thi Khanh Nhu, N., Riordan, D. W., Do Hoang Nhu, T., Thanh, D. P., Thwaites, G., Huong Lan, N. P., et al. (2016). The induction and identification of novel Colistin resistance mutations in *Acinetobacter baumannii* and their implications. *Sci. Rep.* 6:28291. doi: 10.1038/srep28291

Vizcaino, J. A., Csordas, A., Del-Toro, N., Dianes, J. A., Griss, J., Lavidas, I., et al. (2016). 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* 44, D447–D456. doi: 10.1093/nar/gkw880

Wang, S., Yang, Y., Zhao, Y., Zhao, H., Bai, J., Chen, J., et al. (2016). Sub-MIC tylosin inhibits *Streptococcus suis* biofilm formation and results in differential protein expression. *Front. Microbiol.* 7:384. doi: 10.3389/fmicb.2016.00384

Zhou, H., Zhang, T., Yu, D., Pi, B., Yang, Q., Zhou, J., et al. (2011). Genomic analysis of the multidrug-resistant *Acinetobacter baumannii* strain MDR-ZJ06 widely spread in China. *Antimicrob. Agents Chemother.* 55, 4506–4512. doi: 10.1128/AAC.01134-10

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 20 January 2017
doi: 10.3389/fcimb.2017.00007

Quorum Sensing N-acyl Homoserine Lactones-SdiA Suppresses *Escherichia coli-Pseudomonas aeruginosa* Conjugation through Inhibiting *traI* Expression

Yang Lu[1,2†], Jianming Zeng[1†], Binning Wu[3], Shunmei E[1], Lina Wang[1], Renxin Cai[1], Ni Zhang[4], Youqiang Li[1], Xianzhang Huang[1,2], Bin Huang[5] and Cha Chen[1]**

[1] Department of Laboratory Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China, [2] Postdoctoral Mobile Station, Guangzhou University of Chinese Medicine, Guangzhou, China, [3] The Second Clinical College, Guangzhou University of Chinese Medicine, Guangzhou, China, [4] Clinical Microbiology Laboratory, Guangdong Academy of Medical Science and Guangdong General Hospital, Guangzhou, China, [5] Department of Laboratory Medicine, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

OPEN ACCESS

Edited by:
Ghassan M. Matar, American University of Beirut, Lebanon

Reviewed by:
Eric Déziel, Institut National de la Recherche Scientifique, Canada
Shouguang Jin, University of Florida, USA

***Correspondence:**
Bin Huang
huangb3@mail.sysu.edu.cn
Cha Chen
chencha906@163.com

† These authors have contributed equally to this work.

Received: 19 October 2016
Accepted: 05 January 2017
Published: 20 January 2017

Citation:
Lu Y, Zeng J, Wu B, E S, Wang L, Cai R, Zhang N, Li Y, Huang X, Huang B and Chen C (2017) Quorum Sensing N-acyl Homoserine Lactones-SdiA Suppresses Escherichia coli-Pseudomonas aeruginosa Conjugation through Inhibiting traI Expression. Front. Cell. Infect. Microbiol. 7:7. doi: 10.3389/fcimb.2017.00007

Conjugation is a key mechanism for horizontal gene transfer and plays an important role in bacterial evolution, especially with respect to antibiotic resistance. However, little is known about the role of donor and recipient cells in regulation of conjugation. Here, using an *Escherichia coli* (*SM10λπ*)-*Pseudomonas aeruginosa* (*PAO1*) conjugation model, we demonstrated that deficiency of *lasI/rhlI*, genes associated with generation of the quorum sensing signals N-acyl homoserine lactones (AHLs) in *PAO1*, or deletion of the AHLs receptor SdiA in the donor *SM10λπ* both facilitated conjugation. When using another AHLs-non-producing *E. coli* strain *EC600* as recipient cells, deficiency of *sdiA* in donor *SM10λπ* hardly affect the conjugation. More importantly, in the presence of exogenous AHLs, the conjugation efficiency between *SM10λπ* and *EC600* was dramatically decreased, while deficiency of *sdiA* in *SM10λπ* attenuated AHLs-inhibited conjugation. These data suggest the conjugation suppression function of AHLs-SdiA chemical signaling. Further bioinformatics analysis, β-galactosidase reporter system and electrophoretic mobility shift assays characterized the binding site of SdiA on the promoter region of *traI* gene. Furthermore, deletion of *lasI/rhlI* or *sdiA* promoted *traI* mRNA expression in *SM10λπ* and *PAO1* co-culture system, which was abrogated by AHLs. Collectively, our results provide new insight into an important contribution of quorum sensing system AHLs-SdiA to the networks that regulate conjugation.

Keywords: conjugation, N-acyl homoserine lactones, *P. aeruginosa*, SdiA, antibiotic resistance

INTRODUCTION

The acquisition of antibiotic resistance by pathogenic microorganisms is a threat to public health worldwide. Horizontal gene transfer, especially conjugative transfer of plasmids that carry resistance genes, is the primary cause of bacterial antibiotic resistance and—on the larger scale—bacterial evolution (Zatyka and Thomas, 1998; Arthur et al., 2011). The self-transmissible plasmids,

such as the well-studied fertility F-plasmids and IncP plasmid RP4 (also known as RK2), generally present a mobilization (MOB) region which includes the origin of transfer (oriT) and the relaxase gene. The relaxase, identified as being TraI in RP4, initiates conjugation by cleaving the oriT in a site- and strand-specific manner (Carballeira et al., 2014). Other plasmids, termed mobilizable, are incapable of initiating conjugation, but can transfer by using the conjugative apparatus of another plasmid (Zatyka and Thomas, 1998). Mobilizable plasmids are more frequently found in natural environment; therefore, replication and mobilization can be considered as important mechanisms that influence plasmid promiscuity (Fernández-López et al., 2014).

Many Gram-negative bacteria utilize *N*-acyl homoserine lactones (AHLs) as signal molecules to enable individual bacteria to coordinate their behavior in populations; such quorum sensing (QS) enables bacteria to not only sense members of their own species but other species as well (Smith et al., 2011). The essential constituents of QS include a signal producer, or synthase, and a cognate transcriptional regulator that responds to the accumulated signal molecules (Bassler and Losick, 2006). The opportunistic animal and plant pathogen *Pseudomonas aeruginosa* possesses one of the best-studied models of QS, and two different AHL systems, *las* and *rhl*, have been identified (Wagner et al., 2003). In the *las* QS system, the *lasI* gene product directs formation of the diffusible extracellular signal *N*-(3-oxododecanoyl)-L-HSL (3-oxo-C12-HSL), which interacts with LasR to activate a number of virulence genes including the LasA and LasB elastases, exotoxinA, and alkaline protease (Toder et al., 1991; Gambello et al., 1993; Jones et al., 1993; Passador et al., 1993). In the *rhl* system, the *rhlI* gene product catalyzes the synthesis of *N*-butanoyl-L-HSL (C4-HSL). This diffusible signaling molecule, together with RhlR, activates directly some virulence genes like those encoding rhamnolipids and pyocyanin, and represses those genes responsible for assembly and function of the type III secretion system (Bleves et al., 2005; Jimenez et al., 2012). Besides the fact that the *las* and *rhl* systems are hierarchically connected, both *rhlR* and *rhlI* are positively regulated by the *las* system (Wagner et al., 2003). The roles of QS in diverse biological processes, such as virulence, biofilm formation and metabolism in *P. aeruginosa* have attracted research attention (Pearson et al., 1994; Hassett et al., 1999; Whiteley et al., 1999; García-Contreras, 2016). However, as the cell-to-cell communication system, the influence of QS on inter-species conjugation remains largely unknown.

Some organisms, such as *Escherichia*, *Klebsiella*, *Salmonella* and *Shigella* lack AHL synthase and therefore do not produce AHLs; however, they possess a LuxR homolog known as SdiA that can bind AHLs produced by other microorganisms and affect gene expression(Smith and Ahmer, 2003; Yao et al., 2006; Sabag-Daigle et al., 2015). Case et al. described the phenomenon of non-AHLs-producing microorganisms binding and utilizing AHLs produced by other organisms as eavesdropping (Case et al., 2008). Although SdiA can bind to DNA and regulate transcription in the absence of AHLs, the structural studies of SdiA suggest a double mode of action for AHLs on SdiA activity, by increasing both protein stability and DNA-binding affinity (Nguyen et al., 2015; Ishihama et al., 2016). Besides, a neighbor-joining tree analysis revealed that SdiA of *E. coli* did not cluster with the LuxR homologs found in other enterobacterial species, but was closely related to the RhlR of *P. aeruginosa* (Gray and Garey, 2001).

Herein, we clarified the effect of QS on conjugation and investigated the underlying mechanisms by employing a mobilizable plasmid and *E. coli-P. aeruginosa* conjugation model. We found that QS signal molecules produced by *P. aeruginosa* inhibited interspecies conjugation by activating *E. coli* SdiA, resulting in decreased mRNA expression of *traI* in *E. coli*. Blockade of AHL-SdiA signaling using strains deficient in *lasI*, *rhlI* or *sdiA* significantly enhanced conjugative transfer. These findings provide new insight into the regulatory networks of conjugation, and offer novel potential targets for antibiotic resistance.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in **Table 1**. Bacteria were grown in Lysogeny Broth (LB) medium or on LB plates containing 1.5% agar unless otherwise indicated. If required, antibiotics were added at the following final concentrations: ampicillin (Amp, 100 μg/mL), gentamycin (Gm, 30 μg/mL), chloramphenicol (Cm, 20 μg/mL), kanamycin (Kan, 50 μg/mL) and rifampicin (Rif, 50 μg/mL).

Growth Curves

The indicated bacterial strains were cultured in LB overnight (8∼10 h) at 37°C, then diluted to 0.5 MCF (McFarland standard) and 3 mL cultures were grown at 37°C with shaking at 200 rpm. The samples were collected at the indicated time points and the OD_{600} values were determined.

Plasmid Construction

The plasmid pUCP24T was constructed by inserting the *oriT* fragment into pUCP24 (West et al., 1994), which contains a gene cassette (*aacC1*) conferring gentamycin resistance in recipient cells. As a result, pUCP24T is not able to transfer on its own, but can transfer by using the conjugative apparatus of *E. coli SM10λπ*. Details of construction of the plasmids used to delete *sdiA* gene or express SdiA are described in the Supporting Materials and Methods.

Construction of *PAO1 lasI* or *rhlI* and *E. coli SM10λπ sdiA* Deficient Mutants

The phage λ Red recombination system was employed for *sdiA* deletion in *E. coli SM10λπ*, while the *sacB*-based suicide vector system was adapted for knockout of *lasI* or *rhlI* in *PAO1* (Zeng et al., 2016); further details are provided in the Supporting Materials and Methods.

Abbreviations: QS, quorum sensing; AHLs, N-acyl homoserine-lactones; *P. aeruginosa*, *Pseudomonas aeruginosa*; *E. coli*, *Escherichia coli*; qPCR, quantitative real-time PCR.

TABLE 1 | Bacterial strains and plasmids.

Strains/plasmids	Genotype or characteristics	Source
STRAINS		
E. coli SM10λπ	thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir	Simon et al., 1983
E. coli SM10λπ ΔsdiA	Mutants of *E. coli SM10λπ* deficient in *sdiA* gene	This work
E. coli EC600	LacZ-, Nal^{Rr}, Rif^{R}	Our lab
E. coli BW25113	Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), lambda-, rph-1, Δ(rhaD-rhaB)568, hsdR514	Our lab
P. aeruginosa PAO1	Wild-type	Stover et al., 2000
PAO1ΔlasI	Mutants of *PAO1* deficient in *lasI* gene	Our lab Zeng et al., 2016
PAO1ΔrhlI	Mutants of *PAO1* deficient in *rhlI* gene	Our lab Zeng et al., 2016
PLASMIDS		
pKD3	oriR6K, FRT::cat::FRT template plasmid Cm^{R}, Amp^{R}	Datsenko and Wanner, 2000
pKD46	oriR101 repA101ts P-araB-gam-bet-exo Amp^{R}	Datsenko and Wanner, 2000
pCP20	pSC101 temperature-sensitive repliconts, Flp(λ Rp), cI857, Cm^{R}, Amp^{R}	Datsenko and Wanner, 2000
pQF50	Promoterless lacZ reporter plasmid, Amp^{R}	Farinha and Kropinski, 1990
pQF50-*tral*	pQF50 derivative, containing *tral* promoter region, Amp^{R}	This work
pUCP24T	370 bp oriT fragment from pCVD442 cloned into pUCP24, ori1600, Gm^{R}	Philippe et al., 2004

Rif^{R}, Km^{R}, Cm^{R}, Gm^{R}, and Amp^{R} stand for rifampicin, kanamycin, chloramphenicol, gentamycin and ampicillin resistance, respectively.

Conjugation Experiments

For the conjugation assays, the same amount (0.5×10^7 CFU/mL, counted using the Sysmex UF-1000i™ Automated Urine Particle Analyzer; Tokyo, Japan) of mid-logarithmic phase donor (*E. coli SM10λπ* harboring plasmid pUCP24T) and recipient cells (*PAO1* or *EC600*) were mixed in 200 μL LB with or without the indicated HSLs in 96-well plates. After 6 h mating at 37°C, the cultures were vigorously mixed and 30 μL aliquots of each conjugation mixture were spread on LB agar containing 30 μg/mL Gm plus 100 μg/mL Amp for *SM10λπ-PAO1* or 30 μg/mL Gm plus 50 μg/mL Rif for *SM10λπ-EC600* transconjugants. The numbers of transconjugant colonies were counted after overnight incubation at 37°C.

Quantification of HSLs by HPLc-MS/MS

Supernatants of *PAO1*, *PAO1ΔlasI*, and *PAO1ΔrhlI* cultures were collected for HPLC-MS/MS detection of HSLs; full details are provided in the Supporting Materials and Methods.

β-Galactosidase Assays

β-Galactosidase activities were performed on cells in the mid-log phase of growth according to the modified Miller's method (Giacomini et al., 1992). All tests were performed in triplicate.

Electrophoretic Mobility Shift Assays (EMSA)

His-SdiA fusion protein was expressed in *E. coli BL21* (DE3) and purified via Ni-chelating affinity chromatography. Gel shift assays were carried out using the Lightshift Chemiluminescent EMSA kit according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA), details are provided in the Supporting Materials and Methods.

Real-Time PCR

Total RNA was extracted using total RNA isolation reagent (Promega, Madison, WI, USA). Reverse transcription (1 μg of total RNA) was performed with the PrimeScript RT reagent Kit (Takara, Dalian, Liaoning, China). The cDNA was subjected to qPCR on a ViiA™ 7 Dx system (Applied Biosystems, Foster, CA, USA) using SYBR Green qPCR Master Mixes (ThermoFisher Scientific). The expression levels of the target genes were normalized to the expression of the internal control gene (*rpoD*), using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers are listed in Table S1.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. The differences between groups were analyzed using the Student's *t*-test when two groups were compared or one-way ANOVA when more than two groups were compared. All analyses were performed using GraphPad Prism, version 5 (GraphPad Software, Inc., San Diego, CA, USA). All statistical tests were two-sided; $P < 0.05$ was considered statistically significant.

RESULTS

Deficiency of *lasI* or *rhlI* in *P. aeruginosa* Promotes *SM10λπ-PAO1* Conjugation

To elucidate the biological significance of the QS system in *P. aeruginosa* conjugation, we first constructed *lasI* or *rhlI* single gene-deficient mutants, named *PAO1ΔlasI* and *PAO1ΔrhlI*, respectively. In *P. aeruginosa*, *lasI* catalyzes the formation of 3-oxo-C12-HSL, which positively regulates the expression of RhlI. RhlI directs the synthesis of C4-HSL, which subsequently regulates pyocyanin production (O'Loughlin et al., 2013). In this study, despite the existence of *rhlI* in the genome of *PAO1ΔlasI*, both 3-oxo-C12-HSL and C4-HSL were barely detectable in the

conditioned medium of this mutant strain using HPLC-MS/MS analysis. For *PAO1*Δ*rhlI*, the deficiency of *rhlI* in the genome led to an absence of C4-HSL in the conditioned medium of this mutant strain, whereas *lasI* and its product 3-oxo-C12-HSL were present at similar levels as the WT strain (**Figure 1A** and Figure S1). Furthermore, as a result of mutation of the QS system, both *PAO1*Δ*lasI* and *PAO1*Δ*rhlI* lost the ability to express pyocyanin, which could be rescued by exogenous addition of 3-oxo-C12-HSL or C4-HSL (**Figure 1B**). Taken together, these results confirmed the successful creation of *PAO1* strains deficient in *lasI* or *rhlI*.

We subsequently examined the growth and conjugation ability of *PAO1*Δ*lasI* and *PAO1*Δ*rhlI*. Compared to the WT strain, deficiency of *lasI* or *rhlI* hardly affected the growth of *PAO1* (Figure S2), but significantly promoted *SM10λπ-PAO1* conjugation (**Figure 1C**). Furthermore, exogenous 3-oxo-C12-HSL or C4-HSL attenuated the interspecies conjugation ability of *PAO1*Δ*lasI* and *PAO1*Δ*rhlI* (**Figure 1C**). What's more, we counted the amount of donor *SM10λπ* after co-culture with *PAO1*, *PAO1*Δ*lasI* or *PAO1*Δ*rhlI*, and found that there is no difference among the three groups (Figure S3), indicted that the observed effect of quorum sensing on conjugation efficiency was not due to the growth suppressive effect on *SM10λπ*. These data suggested that the QS system may negatively regulate *SM10λπ-PAO1* conjugation.

The Quorum Sensing System of *P. aeruginosa* Inhibits Conjugation by Activating SdiA of *E. coli*

It is well recognized that AHLsq regulate gene transcription via binding to their receptor proteins (LuxR-like proteins). In this conjugation model, in contrast to the recipient cells *PAO1*, the donor *E. coli SM10λπ* cells lack AHL synthase and therefore do not produce AHLs; however, these cells produce a LuxR homolog known as SdiA that can bind AHLs produced by other bacterial species to regulate gene transcription. Given that the conjugative apparatus exist in donor cells, we speculated that *P. aeruginosa*-released AHLs may act on SdiA of *E. coli*. To assess whether SdiA of *E. coli* is involved in the ability of *P. aeruginosa*'s AHLs to inhibit *E. coli-P. aeruginosa* conjugation, we constructed the *sdiA* deficient mutant *SM10λπ*Δ*sdiA*. As expected, deficiency of *sdiA* in *SM10λπ* significantly enhanced *E. coli-P. aeruginosa* conjugation, whereas overexpression of SdiA reversed the phenotype (**Figure 2A**). However, when using a AHLs-non-producing *E. coli* strain *EC600* as the recipient cell, *SM10λπ*Δ*sdiA* did not increase conjugation ability compared to the WT strain (**Figure 2B**). More importantly, the conjugation efficiency of *SM10λπ* and *EC600* significantly decreased in the presence of exogenous 3-oxo-C12-HSL and C4-HSL, while *sdiA* deletion in *SM10λπ* abrogated the effects of AHLs on conjugation (**Figure 2B**), suggesting the inhibitory effect of SdiA on *E. coli-P. aeruginosa* or *SM10λπ-EC600* conjugation is dependent on the presence of AHLs. In addition, growth curves demonstrated that deficiency of *sdiA* in *E. coli* had no influence on cell proliferation (Figures S4, S5), confirming that the regulatory function of SdiA in conjugation in this model was not due to an altered growth rate.

Collectively, these data imply that AHLs produced by *PAO1* may repress *SM10λπ-PAO1* conjugation through binding to SdiA of *E. coli*.

The Interaction between *P. aeruginosa* HSL and *E. coli* SdiA Inhibits the Expression of *traI* in *E. coli*

Mechanisms behind transcription regulation function of SdiA is being disclosed, it seems that genes with specific DNA sequences (SdiA-box) 5′-AAAAG(N8)GAAAA-3′ in the promoter region may be the potential targets of SdiA (Yamamoto et al., 2001). In view of the presence of SdiA-box in the promoter of many SdiA-regulated genes in our bioinformatics analysis (Table S2), we computationally mapped the DNA sequence in the RP4 plasmid to search for conjugation-related genes potentially regulated by SdiA. An SdiA-box sequence (5′-AAGAGcgattgagGAAAA-3′) was identified −317 bp upstream of the *traI* start codon (Figure S6). Subsequently, EMSA assays confirmed the interaction between SdiA and the predicted SdiA-box of the *traI* promoter *in vitro* (**Figures 3A,B**). We therefore further evaluated the role of SdiA in the regulation of *traI* transcription. DNA fragments of *traI* promoter carrying the predicted SdiA-box was cloned upstream of the β -galactosidase gene in the pQF50-promoter reporter. When transformed into *BW25113* (another *E. coli* strain without endogenous β-galactosidase compared to *SM10λπ*), the β-galactosidase activity of pQF50-*traI* was greatly elevated, compared to that of the control, while addition of 3-oxo-C12-HSL and C4-HSL impaired this activity, which was severely attenuated when the *sdiA* was deleted (**Figure 3B**). Intriguing, when AHLs was absent, deletion of *sdiA* hardly affected β-galactosidase activity of pQF50-*traI* (**Figure 3B**), this is consistent with the phenotype shown in **Figure 2**. Compared with the WT strain, *SM10λπ*Δ*sdiA* showed higher mRNA expression of *traI* when cultured with *PAO1* (**Figure 3C**). On the other hand, in the *SM10λπ-PAO1* co-culture system, deficiency of *lasI* or *rhlI* in *PAO1* also led to enhanced expression of *traI* (**Figure 3D**), while supplementation with exogenous 3-oxo-C12-HSL and C4-HSL significantly repressed *traI* expression (**Figure 3E**). These results suggest that repressing *traI* expression in the donor cells may be a critical mechanism behind the inhibitory effect of the AHLs on conjugation.

In summary, we disclosed the cooperative effect of AHLs produced by recipient *P. aeruginosa* cells and SdiA of donor *E. coli* cells in the conjugation regulation. These findings indicate that QS may inhibit conjugation and prevent the excessive dissemination of plasmid.

DISCUSSION

Most recent publications in this field have focused on the regulatory function of QS in virulence and biofilm formation. Here, using *E. coli* (*SM10λπ*) as donor cells and AHLs-producing *P. aeruginosa* (*PAO1*) or non-AHLs producing *E. coli* (*EC600*) as recipient cells, we identified a conjugation-inhibitory effect for

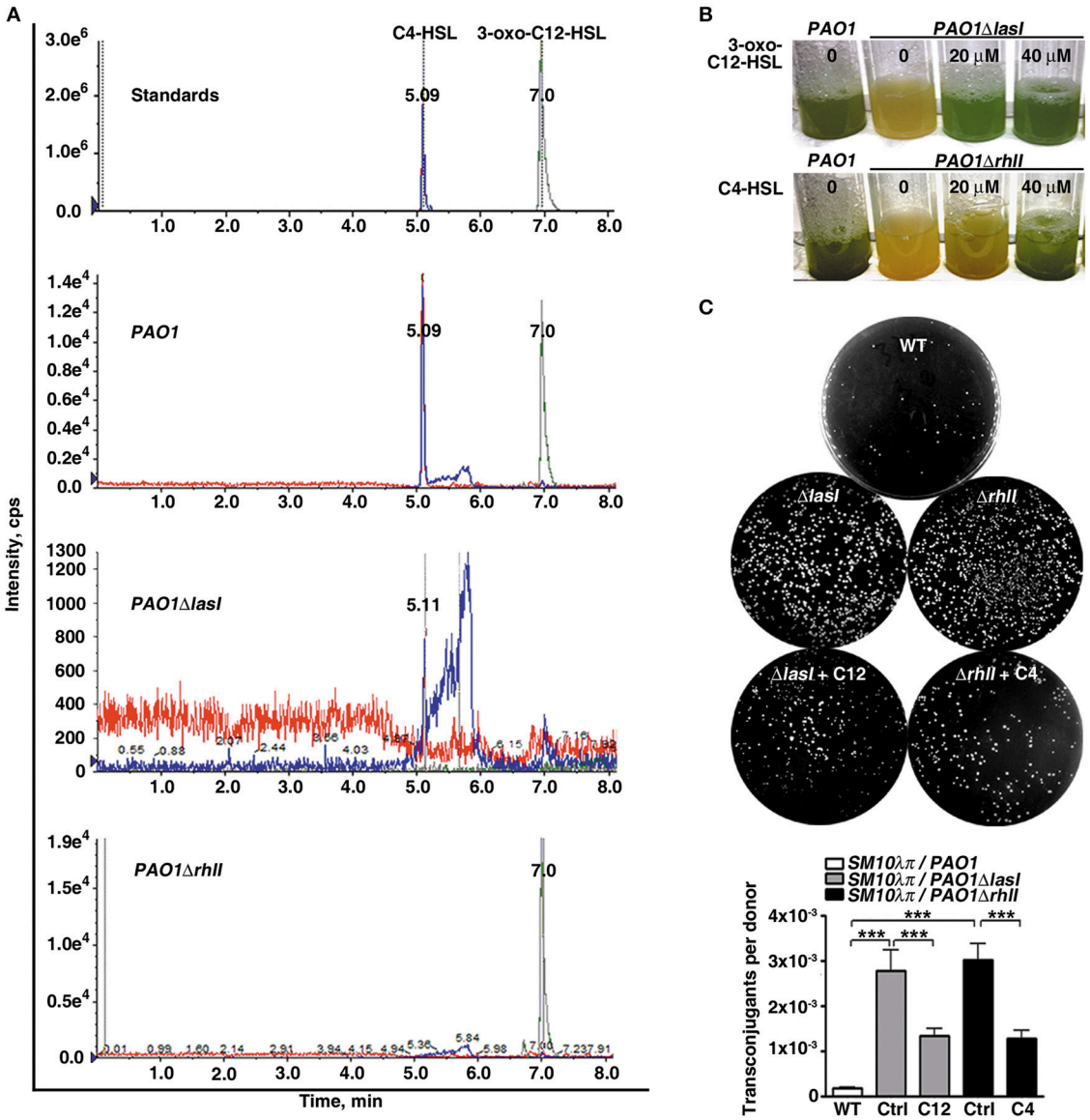


FIGURE 1 | The quorum sensing system of *P. aeruginosa* inhibits conjugation between *P. aeruginosa* and *E. coli*. (A) Deficiency of the AHLs synthase genes *rhlI* or *lasI* in *P. aeruginosa* (*PAO1*) resulted in the absence of C4-HSL or both 3-oxo-C12-HSL and C4-HSL, respectively. The 3-oxo-C12-HSL and C4-HSL in the cell-free supernatants were extracted with ethyl acetate and redissolved in methanol, followed by HPLC-MS/MS analysis. **(B)** Deficiency of *lasI* or *rhlI* in *P. aeruginosa* abolished production of the downstream toxin of *rhlI system* pyocyanin. *PAO1*, *PAO1*Δ*lasI* and *PAO1*Δ*rhlI* were cultured in the presence or absence of 3-oxo-C12-HSL or C4-HSL as indicated for 30 h. **(C)** Deficiency of *lasI* or *rhlI* in *P. aeruginosa* significantly promoted *SM10λπ-PAO1* conjugation; this effect could be abrogated by supplementation with exogenous 3-oxo-C12-HSL or C4-HSL. *SM10λπ* and *PAO1* (10^7 CFU/mL each) were mated at 37°C for 6 h in the presence or absence of 40 μM of C4-HSL or 3-oxo-C12-HSL. Ctrl, control; C12, 3-oxo-C12-HSL; C4, C4-HSL. Values are mean ± SEM of at least three independent experiments; ***$P < 0.001$.

QS based on the following evidence. First, for *SM10λπ* and *PAO1* co-culture system in which AHLs is normally self-sustained, deficiency of the AHLs-producing genes *lasI* or *rhlI* in *PAO1* or the solo AHLs receptor SdiA in *SM10λπ* promoted *SM10λπ-PAO1* conjugation, while supplementation with exogenous 3-oxo-C12-HSL or C4-HSL abrogated the enhanced conjugation ability of *PAO1*Δ*lasI* and *PAO1*Δ*rhlI*. On the other hand, for both non-AHLs producing *SM10λπ* and *EC600* mixed cultures, stimulation with exogenous 3-oxo-C12-HSL and C4-HSL inhibited conjugation, while deletion of *sdiA* in *SM10λπ* attenuated this effect. Conventionally, conjugation is considered to be mainly regulated by the self-transmissible plasmids. While our results indicate that QS system of donor and recipient cells may play a role in conjugation regulation.

Conjugation enables the dissemination of virulence genes and antibiotic resistance genes, which leads to the adaption of bacteria

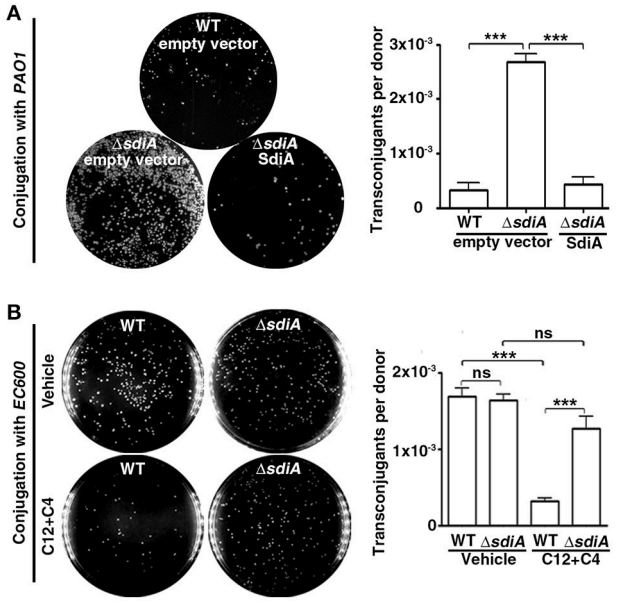


FIGURE 2 | AHL inhibits *SM10λπ-PAO1* conjugation via a mechanism dependent on SdiA of *E. coli*. (A) Deficiency of *sdiA* in *E. coli* significantly promoted *SM10λπ-PAO1* conjugation. *E. coli SM10λπ* and *P. aeruginosa PAO1* (10^7 CFU/mL each) were mated at 37°C for 6 h. **(B)** Deficiency of *sdiA* in *SM10λπ* did not significantly affect *SM10λπ-EC600* conjugation, but could rescue 3-oxo-C12-HSL and C4-HSL (C4/12)-inhibited conjugation. *SM10λπ* was cultured in the presence of DMSO or 40 μM C4-HSL and 3-oxo-C12-HSL for 6 h, followed by conjugation with *EC600*. Values are mean ± SEM of at least three independent experiments; ns, not significant, ***$P < 0.001$.

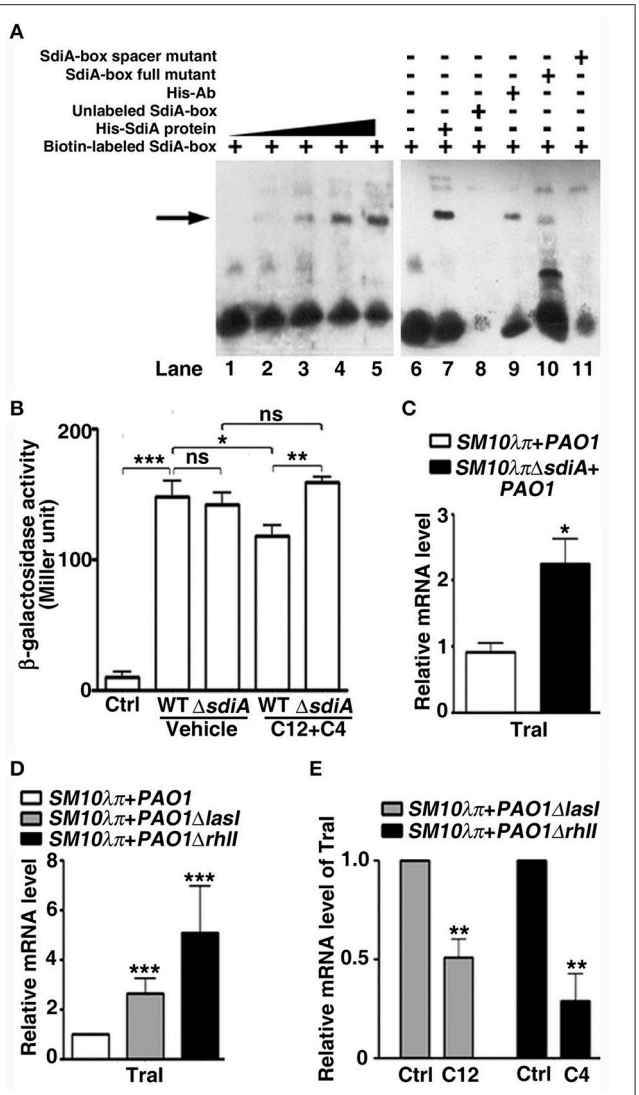


FIGURE 3 | The AHL-SdiA interaction represses the expression of *TraI* in *E. coli*. (A) EMSA verified the interaction of SdiA protein with the putative SdiA-box of the *traI* promoter. Biotin-labeled SdiA-box (50 fmol) was added to each reaction with 0, 2, 4, 8, or 16 pmol *E. coli* His-SdiA fusion protein (lane 1 to lane 5, respectively). The specificity analysis (lane 6 to lane 11) was performed in the presence (–) or absence (+) of His-SdiA fusion protein (8 pmol) and biotin-labeled SdiA-box (50 fmol, lanes 7), 200-fold unlabeled SdiA-box (lanes 8), anti-His antibody (lanes 9), or 200-fold SdiA-box full or spacer mutant competitors (lane 10, 11). **(B)** Activity of β-galactosidase reporters containing the predicted SdiA-box in genomic regions upstream of *traI* under various conditions. *E.coli BW25113* or *E.coli BW25113ΔsdiA* were cultured with *SM10λπ* in the presence of DMSO (Ctrl) or 40 μM C4-HSL and 3-oxo-C12-HSL for 6 h, followed by β-galactosidase activity analysis. **(C)** Deficiency of *sdiA* promoted *traI* expression in *E. coli*. **(D)** Deficiency of *lasI/rhlI* promoted *traI* expression in *E. coli*. For **(C,D)**, 10^7 CFU/mL (each) of the indicated *E. coli* donor and recipient *P. aeruginosa* cells were mated at 37°C for 6 h, followed by real-time PCR analysis. The *rpoD* gene of *E. coli* was used as an internal control. **(E)** Exogenous 3-oxo-C12-HSL or C4-HSL inhibited *traI* expression in *E. coli*. *PAO1ΔlasI* or *PAO1ΔrhlI* were cultured with *SM10λπ* in the presence of DMSO (Ctrl) or 40 μM C4-HSL and 3-oxo-C12-HSL for 6 h, followed by real-time PCR analysis. The *rpoD* gene of *E. coli* was used as an internal control. Ctrl, control; C12, 3-oxo-C12-HSL; C4, C4-HSL. Values are mean ± SEM of at least three independent experiments; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

to new circumstances (Norman et al., 2009). Therefore, the ability to inhibit conjugation may be a potentially efficacious strategy for avoiding the spread of resistance traits. Here, we demonstrate that AHL-SdiA is capable of suppressing conjugation. Most SdiA-expression bacteria, such as *Escherichia*, *Salmonella* and *Shigella* are enterobacteria, while many biological evidences suggest a lack of HSLs in the normal mammalian intestine (Swearingen et al., 2013), despite the presence of AHLs in bovine rumen (Hughes et al., 2010). Thus, although *P. aeruginosa* could be detected in stool sample in our clinical microbiology laboratory, future studies are needed to illuminate the role of AHL-SdiA signaling in pathogenic bacteria communities within the gastrointestinal tract.

To date, many SdiA regulon members have been described (Kanamaru et al., 2000; Wei et al., 2001; Dyszel et al., 2010; Sabag-Daigle et al., 2015). Here we report the identification of SdiA-regulated and AHL-responsive gene *traI* in the plasmid RP4. TraI is reported to function as a relaxase enzyme that creates a nick at the *oriT* of conjugative plasmids, which is required to initiate conjugation (Furuya and Komano, 2000). We discovered a DNA motif recognized by SdiA in the promoter region of the *traI* gene in the plasmid RP4, and the interaction between SdiA and the predicted SdiA-box was validated *in vitro* using an EMSA. However, some SdiA-regulated genes do not have this particular SdiA-box (Dyszel et al., 2010; Swearingen et al., 2013; Abed et al., 2014; Nguyen et al., 2015), there

may be other conjugation-related genes repressed by AHL-SdiA. Moreover, the EMSA was performed without addition of AHLs, so it seems that high concentration of SdiA could bind to *traI* promoter in the absence of AHLs *in vitro* (**Figure 3A**), however, the reporter system (**Figure 3B**) and conjugation experiment (**Figure 2B**) showed that in the absence of AHLs, deletion of *sdiA* hardly affected the promoter activity of *traI*, as well as conjugation frequency *in vivo*. Thus, we proposed AHLs may increase both SdiA protein stability and *traI* promoter-binding affinity to repress *traI* expression.

Despite the advantages of conjugation for bacteria, the introduction of novel genes into the pre-existing, well-tuned genetic background is a source of genetic conflict, and possession of the conjugation-associated machinery also places a burden on the host arising from the energy expended to create and maintain the conjugative apparatus and its associated features (Zatyka and Thomas, 1998; Baltrus, 2013; San Millan et al., 2015). This raises the question of how host bacteria minimize the metabolic cost while obtaining the benefits provided by conjugation. In this study, we found that under normal conditions, when mobilizable plasmid containing a resistance gene was not required by *PAO1* (Table S3), conjugation between *SM10λπ* and *PAO1* was inhibited via the LasI/RhlI-HSL-SdiA pathway. These findings reveal that QS system may play a role in protecting host cells against external conjugative plasmids.

Utilizing ecological data from 2801 samples, Freilich et al. explored the ubiquitous competitive and cooperative interactions between the bacteria within natural communities (Freilich et al., 2011). Nonetheless, revealing more detail of the strategies bacteria adopt for survival in mixed cultures remains a major challenge. The *E. coli-P. aeruginosa* conjugation model has been widely used in studies of bacterial conjugation, and the most prevalent donor strain is *E. coli SMλπ* in which the RP4 plasmid is chromosomally-integrated. Thus, conjugation-associated genes, such as *traI* initially only exist in and are expressed by the *E. coli* (*SM10λπ*) cells, similarly *rhlI* and *lasI* are only expressed by *PAO1*. This makes it easy to detect the expression of these genes in *E. coli* (*SM10λπ*) and *PAO1,* specifically in mixed-cultures. Using this co-culture system, we found that LasI/RhlI and SdiA jointly repressed *traI* expression in *E. coli* and inhibited *SM10λπ-PAO1* conjugation, indicating that the QS system may provide a mechanism of cooperative regulation between bacteria.

In conclusion, the findings of this study highlight the regulatory role for the QS system in conjugation, and expand our understanding of the bacterial communication and defense systems of *P. aeruginosa*.

AUTHOR CONTRIBUTIONS

YL, JZ, XH, BH, and CC designed research; YL, JZ, BW, RC, and NZ performed research; SE and YQL contributed new reagents/analytic tools; YL, JZ, and BW analyzed data; YL, JZ, and CC wrote the paper.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant No. 81572058, 81672081), the Natural Science Foundation of Guangdong Province (Grant No. 2014A030313143) and the Science and Technology Planning Project of Guangdong Province (Grant No. 2016A020215236).

ACKNOWLEDGMENTS

We are very grateful to Prof. B.L. Wanner (Department of Biological Sciences, Purdue University, West Lafayette, USA) for generously providing the λ Red recombination system, Prof. E. Peter Greenberg (Department of Microbiology, University of Washington School of Medicine, Seattle, Washington, USA) for providing pQF50 plasmid and Prof. H. P. Schweizer (Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biological Sciences, Colorado State University, Fort Collins, Colorado, USA) for providing the plasmid pUCP24.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00007/full#supplementary-material

REFERENCES

Abed, N., Grépinet, O., Canepa, S., Hurtado-Escobar, G. A., Guichard, N., Wiedemann, A., et al. (2014). Direct regulation of the pefI-srgC operon encoding the Rck invasin by the quorum-sensing regulator SdiA in *Salmonella Typhimurium*. *Mol. Microbiol.* 94, 254–271. doi: 10.1111/mmi.12738

Arthur, D. C., Edwards, R. A., Tsutakawa, S., Tainer, J. A., Frost, L. S., and Glover, J. N. M. (2011). Mapping interactions between the RNA chaperone FinO and its RNA targets. *Nucleic Acids Res.* 39, 4450–4463. doi: 10.1093/nar/gkr025

Baltrus, D. A. (2013). Exploring the costs of horizontal gene transfer. *Trends Ecol. Evol.* 28, 489–495. doi: 10.1016/j.tree.2013.04.002

Bassler, B. L., and Losick, R. (2006). Bacterially speaking. *Cell* 125, 237–246. doi: 10.1016/j.cell.2006.04.001

Bleves, S., Soscia, C., Nogueira-Orlandi, P., Lazdunski, A., and Filloux, A. (2005). Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 187, 3898–3902. doi: 10.1128/JB.187.11.3898-3902.2005

Carballeira, J. D., González-Pérez, B., Moncalián, G., and De La Cruz, F. (2014). A high security double lock and key mechanism in HUH relaxases controls oriT-processing for plasmid conjugation. *Nucleic Acids Res.* 42, 10632–10643. doi: 10.1093/nar/gku741

Case, R. J., Labbate, M., and Kjelleberg, S. (2008). AHL-driven quorum-sensing circuits: their frequency and function among the *Proteobacteria*. *ISME J.* 2, 345–349. doi: 10.1038/ismej.2008.13

Datsenko, K. A., and Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6640–6645. doi: 10.1073/pnas.120163297

Dyszel, J. L., Soares, J. A., Swearingen, M. C., Lindsay, A., Smith, J. N., and Ahmer, B. M. M. (2010). *E. coli* K-12 and EHEC genes regulated by SdiA. *PLoS ONE* 5:e8946. doi: 10.1371/journal.pone.0008946

Farinha, M. A., and Kropinski, A. M. (1990). Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J. Bacteriol.* 172, 3496–3499. doi: 10.1128/jb.172.6.3496-3499.1990

Fernández-López, C., Bravo, A., Ruiz-Cruz, S., Solano-Collado, V., Garsin, D. A., Lorenzo-Díaz, F., et al. (2014). Mobilizable rolling-circle replicating plasmids from gram-positive bacteria: a low-cost conjugative transfer. *Microbiol. Spectr.* 2, 1–33. doi: 10.1128/microbiolspec.PLAS-0008-2013

Freilich, S., Zarecki, R., Eilam, O., Segal, E. S., Henry, C. S., Kupiec, M., et al. (2011). Competitive and cooperative metabolic interactions in bacterial communities. *Nat. Commun.* 2, 589–595. doi: 10.1038/ncomms1597

Furuya, N., and Komano, T. (2000). Initiation and termination of DNA transfer during conjugation of IncI1 plasmid R64: roles of two sets of inverted repeat sequences within oriT in termination of R64 transfer. *J. Bacteriol.* 182, 3191–3196. doi: 10.1128/JB.182.11.3191-3196.2000

Gambello, M. J., Kaye, S., and Iglewski, B. H. (1993). LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. *Infect. Immun.* 61, 1180–1184.

García-Contreras, R. (2016). Is quorum sensing interference a viable alternative to treat *Pseudomonas aeruginosa* infections? *Front. Microbiol.* 7:1454. doi: 10.3389/fmicb.2016.01454

Giacomini, A., Corich, V., Ollero, F. J., Squartini, A., and Nuti, M. P. (1992). Experimental conditions may affect reproducibility of the beta-galactosidase assay. *FEMS Microbiol. Lett.* 100, 87–90. doi: 10.1111/j.1574-6968.1992.tb05687.x

Gray, K. M., and Garey, J. R. (2001). The evolution of bacterial LuxI and LuxR quorum sensing regulators. *Microbiology* 147, 2379–2387. doi: 10.1099/00221287-147-8-2379

Hassett, D. J., Ma, J. F., Elkins, J. G., McDermott, T. R., Ochsner, U. A., West, S. E., et al. (1999). Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol. Microbiol.* 34, 1082–1093. doi: 10.1046/j.1365-2958.1999.01672.x

Hughes, D. T., Terekhova, D. A., Liou, L., Hovde, C. J., Sahl, J. W., Patankar, A. V., et al. (2010). Chemical sensing in mammalian host-bacterial commensal associations. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9831–9836. doi: 10.1073/pnas.1002551107

Ishihama, A., Shimada, T., and Yamazaki, Y. (2016). Transcription profile of *Escherichia coli*: genomic SELEX search for regulatory targets of transcription factors. *Nucleic Acids Res.* 44, 2058–2074. doi: 10.1093/nar/gkw051

Jimenez, P. N., Koch, G., Thompson, J. A., Xavier, K. B., Cool, R. H., and Quax, W. J. (2012). The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.* 76, 46–65. doi: 10.1128/MMBR.05007-11

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., et al. (1993). The lux autoinducer regulates the production of exoenzyme virulence determinants in Erwinia carotovora and *Pseudomonas aeruginosa*. *EMBO J.* 12, 2477–2482.

Kanamaru, K., Tatsuno, I., Tobe, T., and Sasakawa, C. (2000). SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. *Mol. Microbiol.* 38, 805–816. doi: 10.1046/j.1365-2958.2000.02171.x

Nguyen, Y., Nguyen, N. X., Rogers, J. L., Liao, J., MacMillan, J. B., Jiang, Y., et al. (2015). Structural and mechanistic roles of novel chemical ligands on the SdiA quorum-sensing transcription regulator. *MBio* 6, 2092–2096. doi: 10.1128/mBio.02429-14

Norman, A., Hansen, L. H., and Sørensen, S. J. (2009). Conjugative plasmids: vessels of the communal gene pool. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 364, 2275–2289. doi: 10.1098/rstb.2009.0037

O'Loughlin, C. T., Miller, L. C., Siryaporn, A., Drescher, K., Semmelhack, M. F., and Bassler, B. L. (2013). A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 17981–17986. doi: 10.1073/pnas.1316981110

Passador, L., Cook, J. M., Gambello, M. J., Rust, L., and Iglewski, B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 260, 1127–1130. doi: 10.1126/science.8493556

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H., et al. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 91, 197–201. doi: 10.1073/pnas.91.1.197

Philippe, N., Alcaraz, J. P., Coursange, E., Geiselmann, J., and Schneider, D. (2004). Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. *Plasmid* 51, 246–255. doi: 10.1016/j.plasmid.2004.02.003

Sabag-Daigle, A., Dyszel, J. L., Gonzalez, J. F., Ali, M. M., and Ahmer, B. M. M. (2015). Identification of sdiA-regulated genes in a mouse commensal strain of Enterobacter cloacae. *Front. Cell. Infect. Microbiol.* 5:47. doi: 10.3389/fcimb.2015.00047

San Millan, A., Toll-Riera, M., Qi, Q., and MacLean, R. C. (2015). Interactions between horizontally acquired genes create a fitness cost in *Pseudomonas aeruginosa*. *Nat. Commun.* 6, 6845–6852. doi: 10.1038/ncomms7845

Simon, R., Priefer, U., and Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Nat. Biotechnol.* 9, 784–791. doi: 10.1038/nbt1183-784

Smith, J. L., Fratamico, P. M., and Yan, X. (2011). Eavesdropping by bacteria: the role of SdiA in *Escherichia coli* and *Salmonella enterica* serovar typhimurium quorum sensing. *Foodborne Pathog. Dis.* 8, 169–178. doi: 10.1089/fpd.2010.0651

Smith, J. N., and Ahmer, B. M. M. (2003). Detection of other microbial species by Salmonella: expression of the SdiA regulon. *J. Bacteriol.* 185, 1357–1366. doi: 10.1128/JB.185.4.1357-1366.2003

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., et al. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959–964. doi: 10.1038/35023079

Swearingen, M. C., Sabag-Daigle, A., and Ahmer, B. M. M. (2013). Are there acyl-homoserine lactones within mammalian intestines? *J. Bacteriol.* 195, 173–179. doi: 10.1128/JB.01341-12

Toder, D. S., Gambello, M. J., and Iglewski, B. H. (1991). *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of lasR. *Mol. Microbiol.* 5, 2003–2010. doi: 10.1111/j.1365-2958.1991.tb00822.x

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., and Iglewski, B. H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* 185, 2080–2095. doi: 10.1128/JB.185.7.2080-2095.2003

Wei, Y., Lee, J. M., Smulski, D. R., and LaRossa, R. A. (2001). Global impact of sdiA amplification revealed by comprehensive gene expression profiling of *Escherichia coli*. *J. Bacteriol.* 183, 2265–2272. doi: 10.1128/JB.183.7.2265-2272.2001

West, S. E., Schweizer, H. P., Dall, C., Sample, A. K., and Runyen-Janecky, L. J. (1994). Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* 148, 81–86. doi: 10.1016/0378-1119(94)90237-2

Whiteley, M., Lee, K. M., and Greenberg, E. P. (1999). Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13904–13909. doi: 10.1073/pnas.96.24.13904

Yamamoto, K., Yata, K., Fujita, N., and Ishihama, A. (2001). Novel mode of transcription regulation by SdiA, an *Escherichia coli* homologue of the quorum-sensing regulator. *Mol. Microbiol.* 41, 1187–1198. doi: 10.1046/j.1365-2958.2001.02585.x

Yao, Y., Martinez-Yamout, M. A., Dickerson, T. J., Brogan, A. P., Wright, P. E., and Dyson, H. J. (2006). Structure of the *Escherichia coli* quorum sensing protein SdiA: activation of the folding switch by acyl homoserine lactones. *J. Mol. Biol.* 355, 262–273. doi: 10.1016/j.jmb.2005.10.041

Zatyka, M., and Thomas, C. M. (1998). Control of genes for conjugative transfer of plasmids and other mobile elements. *FEMS Microbiol. Rev.* 21, 291–319. doi: 10.1111/j.1574-6976.1998.tb00355.x

Zeng, J., Zhang, N., Huang, B., Cai, R., Wu, B., E, S., et al. (2016). Mechanism of azithromycin inhibition of HSL synthesis in *Pseudomonas aeruginosa*. *Sci. Rep.* 6:24299. doi: 10.1038/srep24299

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 24 May 2017
doi: 10.3389/fcimb.2017.00209

In vitro Evaluation of the Colistin-Carbapenem Combination in Clinical Isolates of *A. baumannii* Using the Checkerboard, Etest, and Time-Kill Curve Techniques

Micheline A. H. Soudeiha[1], Elias A. Dahdouh[2], Eid Azar[3], Dolla K. Sarkis[1] and Ziad Daoud[3]*

[1] Rodolphe Merieux Laboratory, School of Pharmacy, Saint-Joseph University, Beirut, Lebanon, [2] Animal Health Laboratory, Faculty of Veterinary, Universidad Complutense de Madrid, Madrid, Spain, [3] Clinical Microbiology, Faculty of Medicine and Medical Sciences, University of Balamand, Koura, Lebanon

The worldwide increase in the emergence of carbapenem resistant *Acinetobacter baumannii* (CRAB) calls for the investigation into alternative approaches for treatment. This study aims to evaluate colistin-carbapenem combinations against *Acinetobacter* spp., in order to potentially reduce the need for high concentrations of antibiotics in therapy. This study was conducted on 100 non-duplicate *Acinetobacter* isolates that were collected from different patients admitted at Saint George Hospital-University Medical Center in Beirut. The isolates were identified using API 20NE strips, which contain the necessary agents to cover a panel of biochemical tests, and confirmed by PCR amplification of $bla_{\text{OXA}-51-\text{like}}$. Activities of colistin, meropenem and imipenem against *Acinetobacter* isolates were determined by ETEST and microdilution methods, and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute. In addition, PCR amplifications of the most common beta lactamases contributing to carbapenem resistance were performed. Tri locus PCR–typing was also performed to determine the international clonality of the isolates. Checkerboard, ETEST and time kill curves were then performed to determine the effect of the colistin-carbapenem combinations. The synergistic potential of the combination was then determined by calculating the Fractional Inhibitory Concentration Index (FICI), which is an index that indicates additivity, synergism, or antagonism between the antimicrobial agents. In this study, 84% of the isolates were resistant to meropenem, 78% to imipenem, and only one strain was resistant to colistin. 79% of the isolates harbored $bla_{\text{OXA}-23-\text{like}}$ and pertained to the International Clone II. An additive effect for the colistin-carbapenem combination was observed using all three methods. The combination of colistin-meropenem showed better effects as compared to colistin-imipenem ($p < 0.05$). The colistin-meropenem and colistin-imipenem combinations also showed a decrease of 2.6 and 2.8-fold, respectively in the MIC of colistin ($p < 0.001$). Time kill assays additionally showed synergistic

OPEN ACCESS

Edited by:
Igor Brodsky, University of Pennsylvania, United States

Reviewed by:
David S Weiss, Emory University, United States
Roger Derek Pechous, University of Arkansas for Medical Sciences, United States

***Correspondence:**
Micheline A. H. Soudeiha
micheline.hajjar@hotmail.com;
micheline.hajjar@balamand.edu.lb;
micheline.hajjar@net.usj.edu.lb

Received: *29 January 2017*
Accepted: *09 May 2017*
Published: *24 May 2017*

Citation:
Soudeiha MAH, Dahdouh EA, Azar E, Sarkis DK and Daoud Z (2017) In vitro Evaluation of the Colistin-Carbapenem Combination in Clinical Isolates of A. baumannii Using the Checkerboard, Etest, and Time-Kill Curve Techniques. Front. Cell. Infect. Microbiol. 7:209. doi: 10.3389/fcimb.2017.00209

effects for a few isolates, and no bacterial re-growth was detected following a 24 h incubation. Our study showed that the combination of colistin with carbapenems could be a promising antimicrobial strategy in treating CRAB infections and potentially lowering colistin toxicity related to higher doses used in colistin monotherapy.

Keywords: *Acinetobacter* spp, Checkerboard, time kill curve, perpendicular Etest, International clone

INTRODUCTION

Acinetobacter spp. are organisms that could be found almost ubiquitously in nature. However, some species, especially *Acinetobacter baumannii* and its closely related species, have a great clinical significance in hospital environments since they are often associated with outbreaks and nosocomial infections (Towner, 2009; Howard et al., 2012). Multi-Drug Resistant (MDR) *Acinetobacter baumannii* is being increasingly implicated with infecting critically ill patients. The emergence of Carbapenem Resistant *Acinetobacter baumannii* (CRAB) strains and their detection in several regions across the world makes their treatment increasingly challenging (Towner, 2009; Howard et al., 2012).

A wide range of broad-spectrum antimicrobial agents have been used in the treatment of infections caused by MDR organisms. Of these agents, carbapenems are often resorted to due to their low toxicity and high efficacy (El-Herte et al., 2012). Nonetheless, the overuse and misuse of carbapenems led to an increase in resistance rates against this potent class of antimicrobial agents (El-Herte et al., 2012).

A. baumannii has several innate mechanisms of antibiotic resistance and a heightened ability to acquire resistance to numerous antimicrobial agents. This has led to the emergence of resistance among *A. baumannii* clinical isolates to a wide range of antimicrobial agents, including carbapenems (Howard et al., 2012). Oxacillinases (OXAs) are the most common cause of carbapenem resistance among this species. The intrinsic OXA-51-like is a chromosomally encoded beta-lactamase present in all *A. baumannii* isolates. Although this enzyme by itself does not convey carbapenem resistance, the association of its gene with an insertion sequence drives its over expression and leads to carbapenem resistance (Evans and Amyes, 2014). However, the main cause of carbapenem resistance is the acquisition of other types of oxacillinases (Howard et al., 2012). OXA-23-like, OXA-24-like, OXA-58-like, as well as the recently discovered OXA-143-like and OXA-235-like, are globally associated with the emergence of CRAB (Al Atrouni et al., 2016). Among these OXA families, OXA-23-like was found to be the most prevalent enzyme associated with CRAB infections in Lebanon. OXA-24-like and OXA-58-like have also been reported in this country, but at lower rates (Zarrilli et al., 2008; Al Atrouni et al., 2016). Similar to what is being reported all around the world, CRAB rates are being increasingly reported in Lebanon (Chamoun et al., 2016; Schultz et al., 2016). One Lebanese study showed an increase in the incidence of CRAB isolates from 50.8% in 2011 to 76.5% in 2015 (Al Atrouni et al., 2016; Chamoun et al., 2016). Moreover, International Clone II (ICII) was shown to be the most disseminated clone in this country (Al Atrouni et al., 2016; Schultz et al., 2016).

The high carbapenem resistance rates pose serious therapeutic and infection control challenges, especially since they are associated with high mortality rates and an increase in hospital stay (Zilberberg et al., 2016). Moreover, the lack of effective antibiotics against CRAB isolates led to the re-use of colistin (Neonakis et al., 2011). Colistin (polymyxin E) is a bactericidal antimicrobial agent that was reported to have relatively high levels of toxicity (Bialvaei and Samadi Kafil, 2015). Clinically, two types of colistin are available: Colistin sulfate and colistimethate, which is an inactive form of the drug that is converted to colistin sulfate after hydrolysis (Lim et al., 2010; Bialvaei and Samadi Kafil, 2015; Gurjar, 2015). Though data regarding the pharmacodynamic and pharmacokinetic properties of colistin is scarce, one study from Saudi Arabia showed that 76.1% of the patients treated with high doses of colistimethate developed acute kidney injury (Gurjar, 2015; Omrani et al., 2015). Neurotoxicity during aerolized colistin therapy has also been reported among 2.7% of patients suffering from ventilator associated pneumonia (Abdellatif et al., 2016).

Colistin has shown good *in vitro* activity against gram negative bacilli, including *A. baumannii*. However, resistance to colistin is emerging all across the globe (Ahmed et al., 2016). Colistin resistance in *A. baumannii* has been traced back to the loss or modifications of the Lipopolysaccharide (LPS) molecule (Bialvaei and Samadi Kafil, 2015; Lim et al., 2015; Cheah et al., 2016). A major cause for modifications of the LPS was found to be mutations in the *pmr*CAB operon (Lim et al., 2015), which have been reported to emerge during colistin therapy (Ni et al., 2015). Moreover, high doses of colistin were found to contribute to the emergence of colistin resistance in *Acinetobacter* spp., as well as to the emergence of heteroresistant *Acinetobacter* spp. isolates (Cheah et al., 2016). This, as well as the toxicity of this antimicrobial agent, led to the exploration of the possibility of using colistin in combination with other antimicrobial agents (Batirel et al., 2014). However, no standardized method for the *in vitro* evaluation of combination therapies was established by the CLSI (Tan et al., 2011). Nevertheless, studies have demonstrated a synergistic effect for the combinations of colistin-rifampin and colistin-vancomycin against MDR *A. baumannii* (Ahmed et al., 2016). Interestingly, colistin, vancomycin and rifampin are all antibiotics with relatively high levels of toxicity, whereas carbapenems have low levels of toxicity (Batirel et al., 2014). Nevertheless, few studies have addressed the therapeutic potential of the combination between colistin and carbapenems against CRAB isolates (Daoud et al., 2013). The aim of this study is to characterize *Acinetobacter* spp. isolates obtained from a Lebanese hospital and evaluate the *in vitro* effect of the

colistin–carbapenem combination against these isolates using three different techniques.

MATERIALS AND METHODS

Study Design and Clinical Isolates

This study was carried out at the Saint Georges Hospital-University Medical Center (SGH-UMC). It is a 400-bed tertiary care, educational, medical center located in central Beirut, Lebanon, that attends to around 22,000 admitted patients per year. A total of 100, non-duplicate consecutive isolates were collected from various clinical specimens recovered from 100 patients admitted to this hospital from June 2013 to June 2014. Only one isolate was collected from each patient, regardless of the site of infection it has been recovered from. All the isolates were stored at −80°C in Luria Bertani broth (LB) supplemented with 20% glycerol, and cultured on Mac Conckey agar (Oxoid) prior to testing. An IRB approval (IRB/0/037) was granted from the research committee at SGH-UMC. No written consent from the patients was taken since no interventions were performed.

Identification and Antimicrobial Susceptibility Testing

Conventional biochemical identification was performed using API 20NE strips (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The isolates were identified according to the databases provided by the manufacturers, based on the results of the panel of biochemical tests contained within the strips. Confirmation of the identification of *A. baumannii* was performed through the PCR amplification of $bla_{\text{OXA-51-like}}$ (Le Minh et al., 2015). Susceptibility to different classes of antimicrobial agents was determined by the disc diffusion method (Clinical and Laboratory Standards Institute, 2015). In addition, Minimum Inhibitory Concentrations (MICs) of colistin, meropenem, and imipenem were performed by Etest MIC strips (BD, France, and Liofilchem®) and the broth microdilution methods (Clinical and Laboratory Standards Institute, 2015). Cutoff values were ≤ 2 μg/ ml≥ 4 μg/ ml for colistin and ≤ 2 μg/ ml≥ 8 μg/ ml for meropenem and imipenem (Clinical and Laboratory Standards Institute, 2015). The concentration ranges for the *E*-test were 0.016–256 μg/ ml for colistin and 0.002–32 μg/ ml for the carbapenems (BD, France, and Liofilchem®). The FDA tigecycline breakpoints for *Enterobacteriacaea* were applied to *Acinetobacter* spp. due to lack of breakpoint criteria in the CLSI guidelines (Stein and Babinchak, 2013).

Polymerase Chain Reactions

DNA extraction was performed for all the isolates as described by Zhu et al. (1993). The DNA extracts were preserved at −20°C until used. bla_{IMP}, bla_{VIM}, bla_{NDM}, $bla_{\text{OXA-23-like}}$, $bla_{OXA-24-like}$, $bla_{\text{OXA-48}}$, $bla_{OXA-51-like}$, $bla_{\text{OXA-58-like}}$, bla_{GES}, bla_{KPC}, were tested for by Simplex PCR using the primers listed in **Table 1** (Moubareck et al., 2009; Dallenne et al., 2010;

TABLE 1 | primers used for PCR amplification with their different amplicon size.

Beta-lactamases	Bla gene	Primer direction	Sequence (5'–3')	Size (bp)
CLASS A				
	GES	GES F	ATGCGCTTCATTCACGCAC	863
		GES R	CTATTTGTCCGTGCTCAGGA	
	KPC	KPC F	ATGTCACTGTATCGCCGTCT	881
		KPC R	TTACTGCCCGTTGACGCCCA	
CLASS B				
	IMP	IMP F	CGGCC (G=T) CAGGAG (A=C) G (G=T) CTTT	484
		IMP R	AACCAGTTTTGC(C=T) TTAC(C=T) AT	
	VIM	VIM F	ATTCCGGTCGG(A=G)GAGGTCCG	601
		VIM R	TGTGCTKGAGCAAKTCYAGACCG	
	NDM	NDM F	GGGCCGTATGAGTGATTGC	825
		NDM R	GAAGCTGAGCACCGCATTAG	
CLASS D				
	OXA-23-like	OXA-23-like F	ATGAATAAATATTTTACTTG	821
		OXA-23-like R	TTAAATAATATTCAGCTGTT	
	OXA-24-like	OXA-24-like F	ATACTTCCTATATTCAGCAT	809
		OXA-24-like R	GATTCCAAGATTTCTAGCG	
	OXA-48	OXA48 F	GCTTGATCGCCCTCGATT	281
		OXA48 R	GATTTGCTCCGTGGCCGAAA	
	OXA-51-like	OXA51-like F	TAATGCTTTGATCGGCCTTG	353
		OXA51-like R	TGGATTGCACTTCATCTTGG	
	OXA-58-like	OXA58-Like F	ATGAAATTATTAAAAATATTGAGT	840
		OXA58-like R	ATAAATAATGAAAAACACCCAA	

**R, reverse primer; F, forward primer.*

Ardebili et al., 2014; Rafei et al., 2014). Positive and negative controls for the tested genes were provided from previous studies performed in the lab (Moubareck et al., 2009; Hammoudi et al., 2015).

Clonal Lineage

Tri-locus PCR typing was performed for the isolates that were confirmed as *A. baumannii* through the amplification of $bla_{OXA-51-like}$ after biochemical testing by API 20NE strips (bioMérieux, Marcy l'Etoile, France), in order to determine the clonal relatedness of the *A. baumannii* isolates (Turton et al., 2007). This method consisted of performing two multiplex PCRs targeting different alleles of *ompA, csuE* and $bla_{OXA51-like}$. The different amplification patterns were assigned to an international clone according to the patterns summarized by Karah et al. (2012).

The Checkerboard Technique

The checkerboard technique was performed in triplicate using the combinations of colistin-meropenem and colistin-imipenem for all the 100 isolates obtained as previously described (Daoud et al., 2013). Concentration ranges of 32xMIC to 1/32xMIC for colistin and 8xMIC to 1/8xMIC for carbapenems were prepared in 96-well Microtiter plates (Thermo Scientific™). The concentration ranges were prepared in separate plates and then joined into a single plate so as to have different combinations of the antibiotics in each well. The bacterial inoculum was adjusted to 5×10^5cfu/ml and distributed in all the wells. Two wells were reserved for positive and negative controls, respectively, in each plate. After incubation at 37°C for 24 h, the Fractional Inhibitory Concentration Index (FICI) was calculated using the formula "FICA + FICB = FICI" where "FICA" is the MIC of the drug A in combination/ MIC of the drug A alone; and "FICB" is the MIC of the drug B in combination/ MIC of the drug B alone (Daoud et al., 2013). The sum of FICI was then interpreted as follows: synergy if $\sum$FICI $\leq$ 0.5, additive effect if 0.5 < $\sum$FIC $\leq$ 2, indifference if 2 < $\sum$FIC $\leq$ 4, and antagonism if $\sum$FIC > 4 (Pillaii et al., 2005).

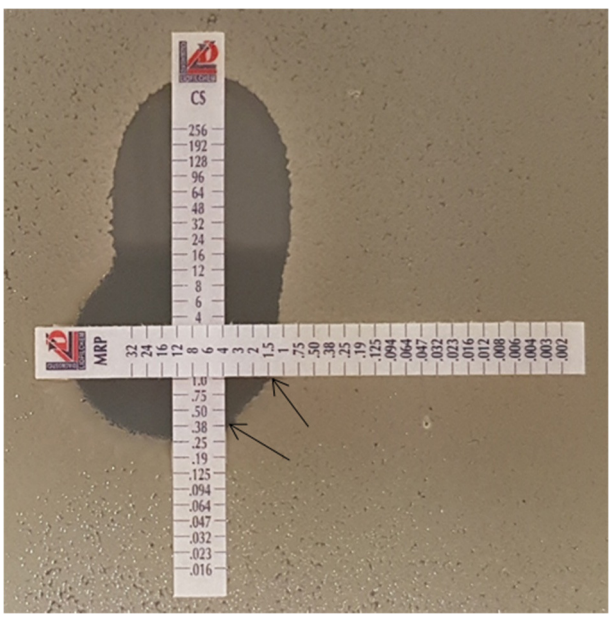


FIGURE 1 | Colistin-meropenem combination using the Etest (90°). Etest strip of colistin with either imipenem or meropenem was placed at 90°crossing at the MIC of the each drug as determined with previous test results. The black arrows indicate the antibiotic concentrations in combination. In this Figure, the MIC of meropenem was determined to be 1.5 μg/ml, and that of colistin in combination was 0.38 μg/ml. CS, colistin; MRP, meropenem.

Perpendicular *E*-test Technique

Perpendicular *E*-tests were carried out in triplicate for 38 representative isolates. In this test, *E*-test strips containing colistin were placed perpendicularly with *E*-test strips containing either imipenem or meropenem over a lawn of the bacterial isolate on Mueller Hinton Agar plates (**Figure 1**). The plates were then incubated at 37°C for 18 h (Doern, 2014). The $\sum$FICI was then calculated and interpreted as described in the previous section (Pillaii et al., 2005).

Time Kill Curve Assay

Time-kill assays were performed in triplicate for 21 representative isolates, as described in the CLSI guidelines (2015). Briefly, concentration ranges of 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125xMIC were prepared in Mueller Hinton Broth (Becton Dickinson, USA) for colistin, imipenem and meropenem alone, and in combination (colistin-imipenem and colistin-meropenem). A 5 x $\times 10^5$cfu/ml inoculum of the tested organism was also prepared and 1,000 μl were used to inoculate the 49 mL of the correspondent broth (containing the antibiotics alone, and in combination). The suspensions were then incubated at 37°C for 8 h with shaking at 200 rpm. An antibiotic-free growth control was also included. At predetermined time points (0, 1, 2, 3, 4, 5, 6, 7, and 8 h), 1,000 μl samples were aseptically acquired for Optical Density (OD) measurements at 580 nm. 100 μl samples were also obtained at these time points, serially diluted, and spread on Mueller Hinton agar plates in order to determine the colony forming units per mL (cfu/mL). Time kill curves were then constructed as a function of time and the results were represented as a difference in log10 between the cfu/mL at 0 and 8 h. A decrease of $\geq$ 3 Log10, as compared to the initial OD or cfu/ml, was indicative of a bactericidal effect (Doern, 2014). Synergistic effects were determined by a decrease of $\geq$ 2 Log10 in OD or cfu/ml when comparing the antibiotics in combination to the most active drug at that time point, while an increase of > 2 Log10 was considered as antagonism (Petersen et al., 2006; Doern, 2014). Additivity/indifference were interpreted as any other outcome that does not meet the criteria for either synergy or antagonism (Petersen et al., 2006).

Statistical Analysis

Normal distribution of the data was tested for using the Kolmogorov-Smirnov test. The data was found to be non-normally distributed and therefore the Wilcoxom and Mann-Whitney tests were carried out for statistical comparisons. *P*-values of less than 0.05 were considered as significant.

RESULTS

Bacterial Isolates and Susceptibility Testing

In this study, 95% of the isolates were identified as pertaining to the *Acinetobacter baumannii-calcoaceticus* complex, 3% as *A. haemolyticus*, 1% as *A. radioresistens*/*A. lwoffii,* and 1% as *A. junii*/*A. johnsonii* by the API 20NE strips (bioMérieux, Marcy l'Etoile, France). Forty percent of the isolates were recovered from patients from the Intensive Care Unit (ICU). Thirty eight percent of the patients were females with a median age of 70 and 62% of the patients were male with a median age of 72.5. As for the site of infection, 62% of the isolates were collected from the respiratory tract, 21% from pus, 5% from blood, and the rest of the isolates were obtained from other sources.

In terms of the susceptibility patterns determined by the disc diffusion method, 84% of the isolates were resistant to meropenem and 78% were resistant to imipenem. 88% of the isolates were classified as MDR since they showed resistance to at least three categories of antimicrobial agents. Although the disc diffusion method showed a 19% resistance to colistin (**Figure 2**), the microdilution method showed that only one isolate was resistant to colistin. This difference in the rate of susceptibility between the two methods could be associated to the limited diffusion of colistin on solid agar (Van Der Heijden et al., 2007). Therefore, for the purposes of this study, the results of the microdilution method were considered for colistin resistance. The MIC_{90} and MIC_{50} of imipenem, meropenem, and colistin are shown in **Table 2**.

Carbapenem Resistance Genes and International Clonality

PCR amplification of the common carbapenemase genes showed that, $bla_{OXA-23-like}$ was present in 79% and $bla_{OXA-24-like}$ in 3% of the isolates. One isolate co-harbored $bla_{OXA-23-like}$ and $bla_{OXA-24-like}$. The chromosomal $bla_{OXA-51-like}$ was detected in 99% of the isolates. The rest of the genes tested for were not detected in any of the isolates.

In terms of international clonality, 82 (86%) of the *A. baumannii* isolates pertained to international clone II (group 1), 6 (6.3%) to group 4, 3 (3.1%) to group 14, 1 (1.05%) to group 10, and 1 (1.05%) to group 8. Two (2.1%) isolates did not pertain to any group.

In vitro Combination Effects

No synergistic effects were detected while combining colistin with the carbapenems using the checkerboard assay. However, additive effects were detected in both combinations where the Ẍ∑FICI of colistin combined with imipenem was 1.169 ± 0.354 and that of colistin with meropenem was 1.109 ± 0.337. The combination of colistin with meropenem showed a better additive effect than the colistin-imipenem combination ($p < 0.05$). The colistin-meropenem combination showed a better additive effect in meropenem resistant isolates when compared to meropenem susceptible isolates ($p < 0.05$) (**Table 3**). The combinations of colistin-imipenem and colistin- meropenem resulted in a decrease of 2.8- and 2.6-folds in the MIC of colistin, respectively. The additivity of the combination of carbapenem-colistin was also evident for the colistin resistant isolate 75.

Perpendicular *E*-tests also showed additive effects of the combinations where the Ẍ∑FICI of colistin combined with imipenem was 1.437± 0.41 and that of colistin with meropenem

TABLE 2 | MIC50 and MIC90 of carbapenems and colistin.

Antibiotics	MIC 50(μg/ml)	MIC 90(μg/ml)
Imipenem	8	32
Meropenem	16	32
Colistin	0.25	1

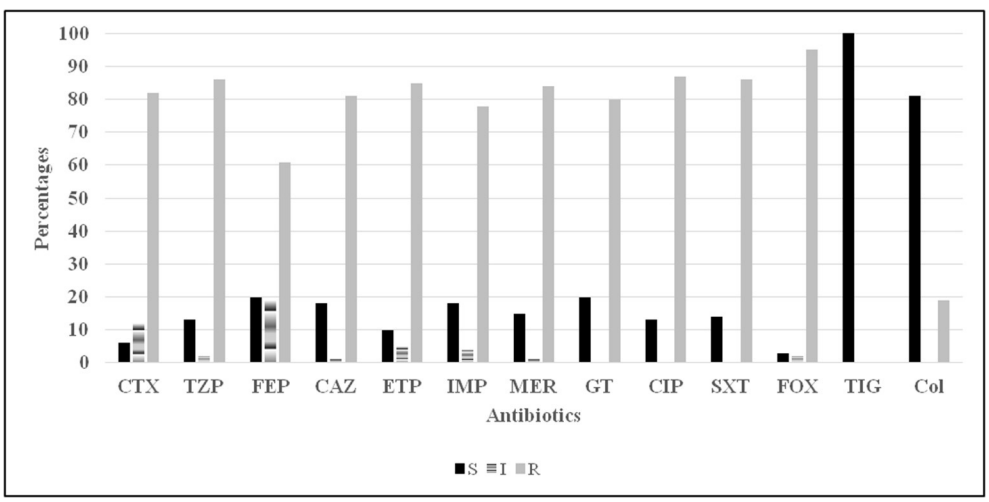


FIGURE 2 | Antibiotic susceptibility (disc diffusion method) profiles for 100 *Acinetobacter* spp. isolates. CTX, cefotaxime; TZP, tazocillin; FEP, cefepime; CAZ, ceftazidime; ETP, ertapenem; Imp, imipenem; MER, meropenem; GT, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim sulfamethoxazole; FOX, cefoxitin; TIG, tigecycline; Col, colistin.

TABLE 3 | Combinations against carbapenem-resistant and susceptible *Acinetobacter* spp.

Comb	Susceptible			Resistant			Impact	*p*-value
	Nb	Mean	SD	Nb	Mean	SD		
Col+Imp	21	1.233	0.387	65	1.138	0.37	Additive	**0.318**
Col+Mer	18	1.251	0.307	78	1.07	0.339	Additive	**0.035**
p-value	0.938			0.14				

Col, colistin; Imp, imipenem; Mer, meropenem; Comb, combination; Nb, number of isolates. Non-bold p-values represent the significance between both combinations among resistant and susceptible isolates, respectively. The bold p-values represent the significance of colistin-imipenem and colistin meropenem combination between the susceptible and resistant isolates, respectively.

TABLE 4 | ΔLog10 values of the cfu/ml of the *Acinetobacter* isolates obtained by the time kill curve assays after incubation, as compared to the initial inoculum.

Antibiotics	Bactericidal effect of colistin and carbapenem alone and in combination (time kill curve)							
	16xMIC	8xMIC	4xMIC	2xMIC	1xMIC	0.5xMIC	0.25xMIC	0.125xMIC
	ΔLog10							
Col	0.429	0.155	0.214	0.441	1.18	1.368	1.621	1.826
Imp	0.325	0.139	0.137	0.121	0.218	1.12	1.914	2.174
Mer	0.413	0.131	0.116	0.282	1.12	1.922	2.054	2.194
Col-Imp	0.437	0.196	0.115	0.066	0.096	0.349	0.915	1.828
Col-Mer	0.403	0.178	0.098	0.195	0.106	0.815	1.06	1.802
p-value	0.528	0.698	0.679	0.103	0.831	0.018	0.112	0.731

Col, colistin; Imp, Imipenem; Mer, meropenem; Col-Imp, colistin imipenem combination, Col-Mer, Colistin meropenem combination. A ΔLog10 value of ≥ 3 Log10 in cfu/ml determines a bactericidal activity.

TABLE 5 | Synergistic effect of the combination of antimicrobial agents against *Acinetobacter* isolates.

Comb	Potential effect of time kill curve							
	16 × 16MIC	8 × 8MIC	4 × 4MIC	2 × 2MIC	1 × 1MIC	0.5 × 0.5MIC	0.25 × 0.25MIC	0.125 × 0.125MIC
	ΔLog10							
Col-Imp	0.001	0.031	−0.11	−0.404	−1.077	−1.059	−0.732	−0.024
Col-Mer	−0.006	0.05	−0.119	−0.274	−0.937	−0.553	−0.554	−0.014
p-value	0.648	0.587	0.735	0.08	0.233	**0.012**	**0.031**	0.879

Comb, combination; Col-Imp, colistin-imipenem combination; Col-Mer, colistin-meropenem combination. A decrease of ≥ 2 Log10 determines a synergistic effect. An increase of > 2 Log10 determines an antagonism. Additivity/indifference is determined for any outcome that does not fit with either synergistic or antagonism effect. Bold values indicates significant difference between combination.

was 1.143 ± 0.50. However, no significant difference between the two combinations was found ($p > 0.05$). The combinations of colistin-imipenem and colistin-meropenem resulted in a decrease of 2.19- and 1.97-folds in the MIC of colistin, respectively.

Time kill curves showed that there was no bactericidal activity detected for all three antibiotics, where all the isolates showed an increase between 0.112 and 2.194 in cfu/ml from time 0 to 8 h. However, the cfu/mL values obtained were generally very low (**Table 4**). A significant bactericidal effect of colistin-imipenem when compared to colistin-meropenem ($p < 0.05$) was determined at 0.5XMIC. Moreover, no bacterial re-growth was detected at the different concentrations of colistin and colistin-carbapenem combinations. It is important to note that, due to limitations that were faced during the experiment, only 13 out of the 21 isolates were tested for at 16XMIC, which could have resulted in obtaining rather high values at this concentration as compared to the other concentrations.

Additive effects of the colistin-carbapenem combinations were observed for all the tested isolates (**Table 5**). Colistin-imipenem combinations showed better additivity than colistin-meropenem at 0.5 × 0.5MIC and 0.25 × 0.25MIC ($p < 0.05$). Synergistic effects for both combinations was detected in isolates 3, 13, 24, and 32 at 2 × 2MIC, 1 × 1MIC, 0.5 × 0.5MIC, and 0.25 × 0.25MIC, respectively (**Figure 3**, **Table 6**) by the time kill assays. No antagonistic effect was detected in this study and only isolate number 71 showed an indifferent effect using the

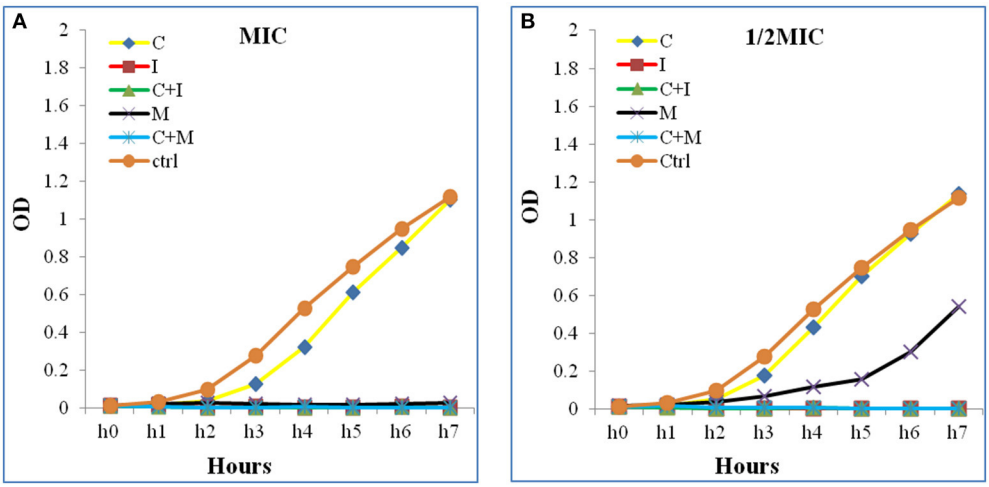


FIGURE 3 | Time kill curve assay chart showing the synergistic effect of a representative strain (A) at 1 × 1MIC and **(B)** at 0.5 × 0.5 MIC. A significant decrease in the bacterial growth in both combinations (C+M and C+I) when compared to Colistin alone at 1 × 1 MIC and 0.5 × 0.5 MIC were detected. The Optical density (OD) is represented as a function of time. C, colistin; I, imipenem; C+I, colistin-imipenem; M, meropenem; C+M, colistin-meropenem; Ctrl, control.

checkerboard technique, but showed additive effects using the other techniques.

DISCUSSION

This study demonstrates the wide dissemination of MDR *Acinetobacter* spp. in SGH-UMC and highlights their dangerous potential in causing severe outbreaks. Our data also falls in line with local Lebanese data in terms of the predominance of IC II and $bla_{OXA-23-like}$ (Al Atrouni et al., 2016).

The results of the combination experiments showed very high rates of additive effects when combining colistin with carbapenems using all three methods. This, however, does not fall in line with findings from another study conducted in Vietnam, where synergistic rates of 68 and 36% have been reported for colistin-meropenem and colistin-imipenem, respectively (Le Minh et al., 2015). The difference in both results determines how isolates pertaining to the same species are able to show diverse responses to antibiotics. This also falls in line with a previous study (Le Minh et al., 2015) that concluded that it is necessary to test *in vitro* combinations prior to *in vivo* use (Le Minh et al., 2015). The difference in results in different regions could be due to the difference in the genetic environments in which the strains are present, and the difference in exposure to antimicrobial agents. These factors could lead to the differential adaptation of the strains in different regions, and the subsequent difference in the interaction of the antimicrobial agents with these strains.

In this study, 99% of the isolates were susceptible to colistin as determined by microdilution method, advocating the use of this antimicrobial agent in monotherapy rather than in combination (Kara et al., 2015). Undoubtedly, the use of monotherapy could lower the chances of toxicity for the patient by avoiding the use of another antibiotic that could cause adverse side effects (Cetin et al., 2013). However, the use of colistin as monotherapy was shown to promote the emergence of heteroresistant *A. baumannii* isolates (Li et al., 2006; Lee et al., 2013) and the development of colistin resistance among other species such as *Pseudomonas aeruginosa*. Moreover, it was shown to promote the proliferation of species that have innate resistance to colistin, such as *Bulkhorderhia cepaciae,* (Cetin et al., 2013). Since these species are considered as highly potent pathogens, they have the potential of causing severe secondary infections following colistin treatment and leave physicians without any viable therapeutic options (Buford et al., 2016; Srinivasan et al., 2016).

Our results showed that combining colistin with carbapenems prevented bacterial re-growth and resulted in 2-fold decreases in colistin MICs. This has very promising implications in terms of using lower doses of colistin in therapy, and thus lowering its potential toxic effects. Moreover, this study showed a better additive effect of the colistin-meropenem combination as compared to the colistin-imipenem combination ($p < 0.05$). A previous study reported that the colistin-meropenem combination is more advantageous as compared to colistin-imipenem in the presence of $bla_{\text{OXA}-23-\text{like}}$ (Daoud et al., 2013). However, in our study, no significant associations between $bla_{\text{OXA}-23-\text{like}}$ and any particular combination were detected ($p > 0.05$). A probable explanation could be associated the higher affinity of meropenem, as compared to imipenem, to penicillin binding proteins in gram negative microorganisms (Le Minh et al., 2015).

With the exception of isolate 71, additivity was uniform while using all three different methods (**Table 6**). This holds true also for the colistin-resistant isolate (isolate 75), showing important implications for the combinations in the treatment of colistin-resistant strains. The additive rates were higher as detected by the checkerboard technique in comparison to the *E*-test ($p < 0.05$). A possible explanation could be the difference in characteristics between the liquid and the solid media that were used for these experiments (Luber et al., 2003). The

TABLE 6 | Comparative table of the three methods used.

Isolates	Comb.	Check.	Etest	Time kill (MICxMIC)							
	Drug A+B	FICI	FICI	16 × 16	8 × 8	4 × 4	2 × 2	1 × 1	0.5 × 0.5	0.25 × 0.25	0.125 × 0.125
				(Δ Log 10)							
3	C+I	1.20	1.16		0.60	−0.34	−0.84	−0.60	**−3.61**	−0.68	0.00
	C+M	1.05	1.60		0.74	−0.74	0.08	−0.60	−1.38	−0.06	0.00
4	C+I	1.04	2.00	0.01	0.05	0.02	−0.10	−0.01	−0.08	−1.37	
	C+M	1.04	2.00	−0.09	−0.23	−0.26	−0.33	−0.24	−0.26	−1.51	
8	C+I	1.41	2.00	−0.01	−0.01	−0.05	0.00	0.01	−0.02	−1.50	
	C+M	1.49	2.00	−0.01	−0.02	−0.03	−0.04	0.02	−0.02	−1.58	
13	C+I	1.41	1.60	0.14	0.05	−0.12	−0.42	**−2.65**	**−2.72**	**−2.71**	
	C+M	1.09	1.60	0.00	0.11	−0.11	−0.13	−0.37	**−2.08**	**−2.16**	
21	C+I	1.23	1.50	−0.01	0.00	−0.04	−0.03	−0.03	−0.02	−0.04	
	C+M	1.23	2.00	0.02	−0.01	0.00	−0.03	−0.03	−0.02	−0.02	
24	C+I	1.05	1.25		0.00	−0.34	−0.20	**−2.86**	−0.22	−0.05	−0.01
	C+M	0.98	1.75		0.40	−0.34	0.00	**−3.20**	−0.14	0.00	0.00
30	C+I	1.83	1.00		0.03	−0.02	−0.04	−0.02	−0.03	0.01	−0.34
	C+M	1.72	1.75		0.00	−0.03	−0.01	−0.02	−0.08	−0.14	0.02
32	C+I	1.05	2.00		0.14	−1.00	**−3.67**	**−3.68**	**−3.06**	−0.24	0.00
	C+M	0.94	1.75		0.00	−1.00	**−3.27**	**−3.68**	−0.13	0.03	0.01
34	C+I	0.99	1.25	−0.01	0.00	0.00	−1.25	−1.49	−1.54	−0.05	
	C+M	0.78	2.00	0.02	0.02	0.04	−1.21	−1.43	−0.16	0.04	
44	C+I	1.05	1.75	0.05	−0.05	−0.03	0.09	−1.33	−1.54	−0.30	
	C+M	0.61	1.16	−0.01	−0.10	−0.03	0.05	−1.33	−0.21	0.07	
48	C+I	1.42	1.40	0.03	0.05	0.02	0.06	0.44	0.00	−1.38	
	C+M	1.13	0.88	0.08	0.07	0.07	0.05	0.53	0.11	−1.43	
51	C+I	1.09	1.50		−0.01	−0.02	0.00	−1.07	−1.45	−1.59	−0.03
	C+M	0.81	2.00		0.01	0.03	0.03	−1.06	−1.42	−0.18	−0.02
63	C+I	1.33	1.40	−0.03	−0.03	−0.04	−0.03	−1.91	−1.46	−0.31	
	C+M	1.53	0.75	0.00	−0.01	0.03	−0.02	−1.33	−0.42	−0.14	
65	C+I	1.46	1.25		−0.04	−0.07	−0.07	−1.67	−1.74	−0.15	0.00
	C+M	1.05	1.25		−0.08	−0.12	−0.11	−1.71	−0.98	0.00	0.02
69	C+I	0.81	1.25	0.07	0.03	0.01	−0.09	−1.51	−1.63	−1.72	
	C+M	0.92	2.00	0.05	0.04	0.00	0.04	−1.38	−1.48	−1.38	
70	C+I	0.68	2.00		−0.05	−0.05	−0.06	−1.37	−1.30	−1.51	−0.03
	C+M	0.72	2.00		0.05	0.02	−0.04	−1.41	−1.48	−1.55	−0.06
71	C+I	2.54	2.00	−0.07	0.08	−0.05	0.09	−0.06	0.05	−0.04	
	C+M	2.09	2.00	0.00	−0.14	−0.11	0.00	−0.05	−0.03	−0.10	
73	C+I	0.67	1.25	−0.14	−0.07	−0.01	−0.66	−1.39	−1.50	−1.58	
	C+M	0.66	1.08	−0.12	−0.11	0.05	−0.64	−1.35	−1.51	−1.57	
75	C+I	0.92	1.00	−0.10	−0.11	−0.12	−0.29	−0.94	−0.02	0.00	
	C+M	0.68	0.85	−0.09	−0.09	−0.11	−0.29	−0.91	0.01	0.05	
78	C+I	0.83	1.60	0.09	0.02	0.00	−0.07	−0.08	−0.04	−0.10	0.00
	C+M	0.93	1.00	0.05	0.06	−0.02	−0.07	−0.07	−0.04	−0.10	
93	C+I	0.70	1.25		0.00	−0.07	−0.93	−0.40	−0.32	−0.07	0.21
		0.76	1.50		0.33	0.16	0.18	−0.07	0.10	0.11	−0.09

C+I, Col-Imipenem; C+M, Col-meropenem. Comb, Combination; Check, Checkerboard. Synergistic effect is determined by ETEST and checkerboard whenever $\sum FICI \leq 0.5$ was detected, and additivity was determined for the range $0.5 < \sum FICI < 2$. All the tested isolates showed additivity in both techniques. The values in bold represent a synergistic effect by time kill curve (< −2). At 2 × 2MIC: Synergistic rate: 4.7% for C+I and C+M respectively, at 1 × 1MIC: synergistic rate: 14.3% for C+I, and 9.5% for C+M, at 0.5 × 0.5MIC synergistic rate 14.3% for C+I, and 4.7% for C+M, at 0.25 × 0.25MIC synergistic rate of 4.7% for C+I and C+M, respectively.

checkerboard technique allows the determination of the optimal concentration of antibiotic capable of killing microorganisms at a determined incubation time (Doern, 2014). However, the time kill assay provides more accurate data regarding the effect of the combinations since the measurements are taken over time, and is able to detect bactericidal activities and bacterial re-growth (Doern, 2014). The higher accuracy of this assay as compared to the others could explain the detection of synergistic effects for some isolates that were not detected in other methods (**Tables 4**, **5**). However, due to the labor-intensive nature of the experiment, it was not carried out for all the isolates included in this study. Nevertheless, our data showed a good agreement between the three methods that were used, as was reported by other studies as well (Petersen et al., 2006; Tan et al., 2011; Cetin et al., 2013). Based on that, the *E*-test method is recommended for use in routine clinical laboratories in case combinations are to be used in therapies since it is easy to perform and provides information regarding the effect of the combination in a rapid manner.

CONCLUSION

In conclusion, a high rate of carbapenem resistance was detected among *A. baumannii* isolates obtained from SGH-UMC. ICII and $bla_{\text{OXA}-23-\text{like}}$ were predominant among these isolates. Combining colistin with carbapenems showed high rates of additive effects using three different methods and resulted in a decrease in colistin MICs. These findings could be very promising in terms of using lesser concentrations of colistin in therapy while combining it with carbapenems and achieve good bacterial clearance rates. However, *in-vivo* experiments are needed before confirming that this combination could be used as a standard therapeutic option.

AUTHOR CONTRIBUTIONS

MS, collected and identified the isolates, performed the susceptibility experiments, PCR experiments, combination techniques, statistical analysis. Was also involved in experiment design and data analysis and drafted the manuscript. ED, performed the tri-locus PCR experiments, clonality analysis, and revision of the manuscript. EA, data analysis. DS, study design and revision of the manuscript. ZD, was involved is study design, data analysis, revision of the manuscript and supervision of all the work performed at the lab.

FUNDING

Saint Joseph University.

ACKNOWLEDGMENTS

The authors would like to thank the laboratory department at SGH-UMC for providing them with the study isolates.

REFERENCES

Abdellatif, S., Trifi, A., Daly, F., Mahjoub, K., Nasri, R., and Lakhal, S. B. (2016). Efficacy and toxicity of aerosolised colistin in ventilator-associated pneumonia: a prospective, randomised trial. *Ann. Intensive Care* 6, 26. doi: 10.1186/s13613-016-0127-7

Ahmed, S. S., Alp, E., Hopman, J., and Voss, A. (2016). Global epidemiology on colistin resistant *Acinetobacter baumannii*. *J. Infect. Dis. Ther.* 4:287. doi:10.4172/2332-0877.1000287

Al Atrouni, A., Hamze, M., Jisr, T., Lemarié, C., Eveillard, M., Joly-Guillou, M.-L., et al. (2016). Wide spread of OXA-23-producing carbapenem-resistant *Acinetobacter baumannii* belonging to clonal complex II in different hospitals in Lebanon. *Int. J. Infect. Dis.* 52, 29–36. doi: 10.1016/j.ijid.2016.09.017

Ardebili, A., Talebi, M., Azimi, L., and Lari, A. R. (2014). Effect of efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone on the minimum inhibitory concentration of ciprofloxacin in *Acinetobacter baumannii* clinical isolates. *Jundishapur J. Microbiol.* 7:e8691. doi: 10.5812/jjm.8691

Batirel, A., Balkan, I., Karabay, O., Agalar, C., Akalin, S., Alici, O., et al. (2014). Comparison of colistin–carbapenem, colistin–sulbactam, and colistin plus other antibacterial agents for the treatment of extremely drug-resistant *Acinetobacter baumannii* bloodstream infections. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 1311–1322. doi: 10.1007/s10096-014-2070-6

Bialvaei, A. Z., and Samadi Kafil, H. (2015). Colistin, mechanisms and prevalence of resistance. *Curr. Med. Res. Opin.* 31, 707–721. doi: 10.1185/03007995.2015.1018989

Buford, V. R., Kumar, V., and Kennedy, B. R. (2016). Relationship of various infection control interventions to the prevalence of multidrug-resistant *Pseudomonas aeruginosa* among US hospitals. *Am. J. Infect. Control* 44, 381–386. doi: 10.1016/j.ajic.2015.10.033

Cetin, E. S., Tekeli, A., Ozseven, A. G., Us, E., and Aridogan, B. C. (2013). Determination of *in vitro* activities of polymyxin B and rifampin in combination with ampicillin/sulbactam or cefoperazone/sulbactam against multidrug-resistant *Acinetobacter baumannii* by the *E*-test and checkerboard methods. *Jpn. J. Infect. Dis.* 66, 463–468. doi: 10.7883/yoken.66.463

Chamoun, K., Farah, M., Araj, G., Daoud, Z., Moghnieh, R., Salameh, P., et al. (2016). Surveillance of antimicrobial resistance in Lebanese hospitals: retrospective nationwide compiled data. *Int. J. Infect. Dis.* 46, 64–70. doi: 10.1016/j.ijid.2016.03.010

Cheah, S.-E., Li, J., Tsuji, B. T., Forrest, A., Bulitta, J. B., and Nation, R. L. (2016). Colistin and polymyxin B dosage regimens against *Acinetobacter baumannii*: differences in activity and the emergence of resistance. *Antimicrob. Agents Chemother.* 60, 3921–3933. doi: 10.1128/AAC.02927-15

Clinical and Laboratory Standards Institute (2015). *Performance Standards For Antimicrobial Susceptibility. Twenty-Five Informational Supplement* M100–S25. Wayne, PA: CLSI.

Dallenne, C., Da Costa, A., Decré, D., Favier, C., and Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. *J. Antimicrob. Chemother.* 65, 490–495. doi: 10.1093/jac/dkp498

Daoud, Z., Mansour, N., and Masri, K. (2013). Synergistic combination of carbapenems and colistin against *P. aeruginosa* and *A. baumannii*. *Open J. Med. Microbiol.* 3, 253–258. doi: 10.4236/ojmm.2013.34038

Doern, C. D. (2014). When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. *J. Clin. Microbiol.* 52, 4124–4128. doi: 10.1128/JCM.01121-14

El-Herte, R. I., Kanj, S. S., Matar, G. M., and Araj, G. F. (2012). The threat of carbapenem-resistant Enterobacteriaceae in Lebanon: an update on the regional and local epidemiology. *J. Infect. Public Health* 5, 233–243. doi: 10.1016/j.jiph.2012.02.003

Evans, B. A., and Amyes, S. G. (2014). OXA β-lactamases. *Clin. Microbiol. Rev.* 27, 241–263. doi: 10.1128/CMR.00117-13

Gurjar, M. (2015). Colistin for lung infection: an update. *J. Intensive Care* 3:3. doi: 10.1186/s40560-015-0072-9

Hammoudi, D., Moubareck, C. A., Hakime, N., Houmani, M., Barakat, A., Najjar, Z., et al. (2015). Spread of imipenem-resistant *Acinetobacter baumannii* co-expressing OXA-23 and GES-11 carbapenemases in Lebanon. *Int. J. Infect. Dis.* 36, 56–61. doi: 10.1016/j.ijid.2015.05.015

Howard, A., O'Donoghue, M., Feeney, A., and Sleator, R. D. (2012). *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence* 3, 243–250. doi: 10.4161/viru.19700

Kara, I., Yildirim, F., Bilaloglu, B., Karamanlioglu, D., Kayacan, E., Dizbay, M., et al. (2015). Comparison of the efficacy of colistin monotherapy and colistin combination therapies in the treatment of nosocomial pneumonia and ventilator-associated pneumonia caused by *Acinetobacter baumannii*: article. *S. Afr. J. Crit. Care* 31, 51–58. doi: 10.7196/SAJCC.2015.v31i2.246

Karah, N., Sundsfjord, A., Towner, K., and Samuelsen, Ø (2012). Insights into the global molecular epidemiology of carbapenem non-susceptible clones of *Acinetobacter baumannii*. *Drug Resist. Updat.* 15, 237–247. doi: 10.1016/j.drup.2012.06.001

Lee, H. J., Bergen, P. J., Bulitta, J. B., Tsuji, B., Forrest, A., Nation, R. L., et al. (2013). Synergistic activity of colistin and rifampin combination against multidrug-resistant *Acinetobacter baumannii* in an *in vitro* pharmacokinetic/pharmacodynamic model. *Antimicrob. Agents Chemother.* 57, 3738–3745. doi: 10.1128/AAC.00703-13

Le Minh, V., Nhu, N. T. K., Phat, V. V., Thompson, C., Lan, N. P. H., Nga, T. V. T., et al. (2015). *In vitro* activity of colistin in antimicrobial combination against carbapenem-resistant *Acinetobacter baumannii* isolated from patients with ventilator-associated pneumonia in Vietnam. *J. Med. Microbiol.* 64, 1162–1169 doi: 10.1099/jmm.0.000137

Li, J., Rayner, C. R., Nation, R. L., Owen, R. J., Spelman, D., Tan, K. E., et al. (2006). Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 50, 2946–2950. doi: 10.1128/AAC.00103-06

Lim, L. M., Ly, N., Anderson, D., Yang, J. C., Macander, L., Jarkowski, A., et al. (2010). Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. *Pharmacotherapy* 30, 1279–1291. doi: 10.1592/phco.30.12.1279

Lim, T. P., Ong, R. T.-H., Hon, P.-Y., Hawkey, J., Holt, K. E., Koh, T. H., et al. (2015). Multiple genetic mutations associated with polymyxin resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 59, 7899–7902. doi: 10.1128/AAC.01884-15

Luber, P., Bartelt, E., Genschow, E., Wagner, J., and Hahn, H. (2003). Comparison of broth microdilution, E Test, and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni* and *Campylobacter coli*. *J. Clin. Microbiol.* 41, 1062–1068. doi: 10.1128/JCM.41.3.1062-1068.2003

Moubareck, C., Brémont, S., Conroy, M.-C., Courvalin, P., and Lambert, T. (2009). GES-11, a novel integron-associated GES variant in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53, 3579–3581. doi: 10.1128/AAC.00072-09

Neonakis, I. K., Spandidos, D. A., and Petinaki, E. (2011). Confronting multidrug-resistant *Acinetobacter baumannii*: a review. *Int. J. Antimicrob. Agents* 37, 102–109. doi: 10.1016/j.ijantimicag.2010.10.014

Ni, W., Shao, X., Di, X., Cui, J., Wang, R., and Liu, Y. (2015). *In vitro* synergy of polymyxins with other antibiotics for *Acinetobacter baumannii*: a systematic review and meta-analysis. *Int. J. Antimicrob. Agents* 45, 8–18. doi: 10.1016/j.ijantimicag.2014.10.002

Omrani, A. S., Alfahad, W. A., Shoukri, M. M., Baadani, A. M., Aldalbahi, S., Almitwazi, A. A., et al. (2015). High dose intravenous colistin methanesulfonate therapy is associated with high rates of nephrotoxicity; a prospective cohort study from Saudi Arabia. *Ann. Clin. Microbiol. Antimicrob.* 14:3. doi: 10.1186/s12941-015-0062-8

Petersen, P. J., Labthavikul, P., Jones, C. H., and Bradford, P. A. (2006). *In vitro* antibacterial activities of tigecycline in combination with other antimicrobial agents determined by chequerboard and time-kill kinetic analysis. *J. Antimicrob. Chemother.* 57, 573–576. doi: 10.1093/jac/dki477

Pillaii, S., Moellering, R., and Elliopoulos, G. (2005). "Antimicrobial combination", in *Antibiotics in Laboratory Medicine* eds V. Lorian (Baltimore, MD: Lippincott Williams & Wilkins), 365–440.

Rafei, R., Dabboussi, F., Hamze, M., Eveillard, M., Lemarié, C., Mallat, H., et al. (2014). First report of bla NDM-1-producing *Acinetobacter baumannii* isolated in Lebanon from civilians wounded during the Syrian war. *Int. J. Infect. Dis.* 21, 21–23. doi: 10.1016/j.ijid.2014.01.004

Schultz, M. B., Thanh, D. P., Do Hoan, N. T., Wick, R. R., Ingle, D. J., Hawkey, J., et al. (2016). Repeated local emergence of carbapenem-resistant *Acinetobacter baumannii* in a single hospital ward. *Microb. Genom.* 2:e000050. doi: 10.1099/mgen.0.000050

Srinivasan, S., Arora, N., and Sahai, K. (2016). Report on the newly emerging nosocomial *Burkholderia cepacia* in a tertiary hospital. *Med. J. Armed Forces India* 72, S50–S53. doi: 10.1016/j.mjafi.2016.03.003

Stein, G. E., and Babinchak, T. (2013). Tigecycline: an update. *Diagn. Microbiol. Infect. Dis.* 75, 331–336. doi: 10.1016/j.diagmicrobio.2012.12.004

Tan, T. Y., Lim, T. P., Lee, W. H. L., Sasikala, S., Hsu, L. Y., and Kwa, A. L.-H. (2011). *In vitro* antibiotic synergy in extensively drug-resistant *Acinetobacter baumannii*: the effect of testing by time-kill, checkerboard, and Etest methods. *Antimicrob. Agents Chemother.* 55, 436–438. doi: 10.1128/AAC.00850-10

Towner, K. (2009). Acinetobacter: an old friend, but a new enemy. *J. Hosp. Infect.* 73, 355–363. doi: 10.1016/j.jhin.2009.03.032

Turton, J., Gabriel, S., Valderrey, C., Kaufmann, M., and Pitt, T. (2007). Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clin. Microbiol. Infect.* 13, 807–815. doi: 10.1111/j.1469-0691.2007.01759.x

Van Der Heijden, I. M., Levin, A. S., De Pedri, E. H., Fung, L., Rossi, F., Duboc, G., et al. (2007). Comparison of disc diffusion, Etest and broth microdilution for testing susceptibility of carbapenem-resistant *P. aeruginosa* to polymyxins. *Ann. Clin. Microbiol. Antimicrob.* 6:8. doi: 10.1186/1476-0711-6-8

Zarrilli, R., Vitale, D., Di Popolo, A., Bagattini, M., Daoud, Z., Khan, A. U., et al. (2008). A plasmid-borne blaOXA-58 gene confers imipenem resistance to *Acinetobacter baumannii* isolates from a Lebanese hospital. *Antimicrob. Agents Chemother.* 52, 4115–4120. doi: 10.1128/AAC.00366-08

Zhu, H., Qu, F., and Zhu, L.-H. (1993). Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Res.* 21, 5279 doi: 10.1093/nar/21.22.5279

Zilberberg, M. D., Nathanson, B. H., Sulham, K., Fan, W., and Shorr, A. F. (2016). Multidrug resistance, inappropriate empiric therapy, and hospital mortality in *Acinetobacter baumannii* pneumonia and sepsis. *Crit. Care* 20, 221 doi: 10.1186/s13054-016-1392-4

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 15 February 2017
doi: 10.3389/fcimb.2017.00041

Human Salivary Protein Histatin 5 Has Potent Bactericidal Activity against ESKAPE Pathogens

Han Du[1], *Sumant Puri*[1], *Andrew McCall*[1], *Hannah L. Norris*[1], *Thomas Russo*[2, 3, 4, 5] *and Mira Edgerton*[1]*

[1] Department of Oral Biology, School of Dental Medicine, University at Buffalo, Buffalo, NY, USA, [2] Veterans Administration Western New York Healthcare System, Buffalo, NY, USA, [3] The Department of Medicine, University at Buffalo, Buffalo, NY, USA, [4] Department of Microbiology and Immunology, University at Buffalo, Buffalo, NY, USA, [5] The Witebsky Center for Microbial Pathogenesis, University at Buffalo, Buffalo, NY, USA

ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens have characteristic multiple-drug resistance and cause an increasing number of nosocomial infections worldwide. Peptide-based therapeutics to treat ESKAPE infections might be an alternative to conventional antibiotics. Histatin 5 (Hst 5) is a salivary cationic histidine-rich peptide produced only in humans and higher primates. It has high antifungal activity against *Candida albicans* through an energy-dependent, non-lytic process; but its bactericidal effects are less known. We found Hst 5 has bactericidal activity against *S. aureus* (60–70% killing) and *A. baumannii* (85–90% killing) in 10 and 100 mM sodium phosphate buffer (NaPB), while killing of >99% of *P. aeruginosa*, 60–80% *E. cloacae* and 20–60% of *E. faecium* was found in 10 mM NaPB. Hst 5 killed 60% of biofilm cells of *P. aeruginosa*, but had reduced activity against biofilms of *S. aureus* and *A. baumannii*. Hst 5 killed 20% of *K. pneumonia* biofilm cells but not planktonic cells. Binding and uptake studies using FITC-labeled Hst 5 showed *E. faecium and E. cloacae* killing required Hst 5 internalization and was energy dependent, while bactericidal activity was rapid against *P. aeruginosa* and *A. baumannii* suggesting membrane disruption. Hst 5-mediated killing of *S. aureus* was both non-lytic and energy independent. Additionally, we found that spermidine conjugated Hst 5 (Hst5-Spd) had improved killing activity against *E. faecium*, *E. cloacae,* and *A. baumannii.* Hst 5 or its derivative has antibacterial activity against five out of six ESKAPE pathogens and may be an alternative treatment for these infections.

Keywords: ESKAPE, antimicrobial peptide, Histatin 5, *Candida albicans*, bactericidal activity

OPEN ACCESS

Edited by:
Ghassan M. Matar, American University of Beirut, Lebanon

Reviewed by:
Gill Diamond, University of Florida, USA
Jens Kreth, Oregon Health & Science University, USA

***Correspondence:**
Mira Edgerton
edgerto@buffalo.edu

Received: *13 October 2016*
Accepted: *03 February 2017*
Published: *15 February 2017*

Citation:
Du H, Puri S, McCall A, Norris HL, Russo T and Edgerton M (2017) Human Salivary Protein Histatin 5 Has Potent Bactericidal Activity against ESKAPE Pathogens. Front. Cell. Infect. Microbiol. 7:41. doi: 10.3389/fcimb.2017.00041

INTRODUCTION

Bacteria causing nosocomial infections are increasingly becoming drug resistant, posing a serious health concern, especially in the Intensive Care Units (ICUs) and in surgical wards. A recent report estimated a total of 722,000 of such drug-resistant infections, with an astonishing 75,000 resulting in deaths (Magill et al., 2014). A group of bacterial pathogens including *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa*, and *Enterobacter* species (referred to as ESKAPE pathogens) have been of particular concern in

this regard (Rice, 2010). Two-thirds of all healthcare-associated infections are ESKAPE related (Boucher et al., 2009), and many of these bacteria use multiple drug resistance mechanisms to "eskape" killing by both conventional and some newer generation antibiotics (Rice, 2008).

Most nosocomial infections result from an exogenous inoculum including the hospital environment and medical personnel, resulting in colonization of ESKAPE pathogens within various patient niches. *E. faecium, K. pneumoniae*, *P. aeruginosa,* and *Enterobacter* species are common residents of mucosal surfaces such as the oral cavity and the gastrointestinal tract (Keller et al., 1998; Podschun and Ullmann, 1998; Rice, 2010; Vu and Carvalho, 2011). *S. aureus* is a skin commensal and *A. baumanni* has been known to have comparatively long survival rates on epithelial surfaces (e.g., skin; Houang et al., 1998; Coates et al., 2014). Also, *P. aeruginosa* and *K. pneumonia* were detected in 50 and 31%, respectively, of HIV-positive patients' salivas in hospital settings (Lopes et al., 2015). Thus, colonization of the oral cavity with *K. pneumoniae*, *P. aeruginosa,* and *Enterobacter species* can serve as a potential inoculum source for pneumonia (Sands et al., 2016). Furthermore, *Candida albicans*, an oral commensal fungus also present in the oral cavity, can contribute to a more robust *P. aeruginosa* lung infection if the two are present together (Lindsay and Hogan, 2014).

Salivary innate immunity is the first line of defense against transient and pathobionts in the oral cavity (Salvatori et al., 2016). This is illustrated by the example of salivary Histatin 5 (Hst 5), a cationic protein that has strong fungicidal activity against *C. albicans* (Puri and Edgerton, 2014). Changes in the levels of Hst 5 associated with immunodeficiency may increase susceptibility to oral candidiasis caused by *C. albicans* (Khan et al., 2013). However, Hst 5 was shown to be largely ineffective against oral commensal bacteria as well as cariogenic or periodontal pathogens (Devine and Hancock, 2002; Groenink et al., 2003; Dale and Fredericks, 2005).

However, antimicrobial activity of Hst 5 against the ESKAPE pathogens has never been examined. Hst 5-mediated candidacidal activity is an energy-dependent, non-lytic process, where multiple intracellular targets are affected after Hst 5 has been transported to the cytosol in an energy dependent manner (Puri and Edgerton, 2014). Given the fact that most ESKAPE pathogens are not present in large numbers in the oral cavity of healthy humans, and do not cause any known oral diseases, we hypothesized that salivary Hst 5 may play a substantial role in keeping at least some of the ESKAPE pathogens in check within the oral environment. Here we show for the first time that Hst 5 demonstrates killing activity against all ESKAPE pathogens except *K. pneumoniae*. Killing against some ESKAPE pathogens was lytic in nature; however, as for *C. albicans*, more than one mechanism of killing seems to be involved in Hst 5 activity against ESKAPE bacteria.

MATERIALS AND METHODS

Strains, Culture Conditions, and Peptides

All ESKAPE strains used in this study are clinical isolates and are listed in **Table 1**. These isolates were collected over the past 20 years directly from the clinical microbiology laboratory at the University at Buffalo, which performed strain identification by approved laboratory methodology. Isolates from de-identified plates were frozen and stored at −80°C. *Enterococcus faecium* and *Staphylococcus aureus* were grown in Trypticase soy broth (Sigma-Aldrich; TSB) or TSB agar. All other strains were cultured in Luria-Bertani (LB; BD Biosciences) broth or on LB agar. The incubation temperature for all strains was 37°C. Histatin 5 (Hst 5), FITC-labeled Hst 5 (F-Hst 5), and spermidine conjugated Histatin 5 (Hst 5-Spd) were synthesized by Genemed Synthesis Inc. (San Antonio, Texas). Spermidine was purchased from Sigma-Aldrich.

TABLE 1 | ESKAPE strains used in this study.

Species	Strain #	Gram staining	Source*
Enterococcus faecium	94-346-1139	+	Buffalo, NY
Staphylococcus aureus	299-0432	+	Buffalo, NY
Staphylococcus aureus (MRSA)	94-292-1348	+	Buffalo, NY
Klebsiella pneumoniae	Montreal #33	-	Montreal, Canada
Acinetobacter baumannii	AB307-0294	-	Buffalo, NY (Adams et al., 2008)
Acinetobacter baumannii	HUMC1	-	(Luo et al., 2012)
Pseudomonas aeruginosa	94-323-0635	-	Buffalo, NY
Pseudomonas aeruginosa	PAO1	-	Sutton lab, Buffalo, NY
Enterobacter cloacae	94-293-0624	-	Buffalo, NY

**Isolates from the University at Buffalo were collected over the past 20 years directly from the clinical microbiology laboratory, which performed strain identification by approved laboratory methodology.*

Bactericidal Assay

Bactericidal assays were performed by microdilution plate method as described for candidacidal assays (Jang et al., 2010), with some modifications. Briefly, a single colony of each strain was inoculated into 10 mL of media and grown for 16 h at 37°C, cultures were diluted to an $OD_{600} = 0.1$ in fresh media and incubated with shaking at 37°C to mid-log phase ($OD_{600} \approx 1.0$). Cultures were spun down at 1800 × g for 3 min and washed three times with 10 mM sodium phosphate buffer (pH 7.4; NaPB). Cells were re-suspended (10^7 cells/mL) in 200 μL NaPB (control) or in NaPB containing peptides (30 μM); then incubated at 37°C (except for *S. aureus* that was incubated at room temperature for optimal growth). Aliquots were removed after 1 min, 1 h and 5 h incubation, then were serially diluted with phosphate buffered saline (PBS; 137 mM NaCl, 10 mM sodium phosphate, 2.7 mM KCl, pH of 7.4); plated and incubated for 24 h at 37°C to visualize surviving CFUs. Assays were performed in triplicate. Percentage killing was calculated as [1 − number of colonies from peptide-treated cells/mean number of colonies from control cells] × 100%. Bactericidal assays were also performed in 100 mM NaPB and PBS.

Live Cell Imaging of F-Hst 5 and PI Uptake by Time-Lapse Confocal Microscopy

Confocal microscopy was performed using live cells of six ESKAPE strains attached to chambered borosilicate coverglass

(Lab-Tek #155411, 8 chamber). Cells in mid-log phase were collected and washed three times and re-suspended in 10 mM NaPB. Cells (2×10^7 in 200 μL NaPB) were added to each well, allowed to settle for 15 min, then propidium iodide (PI; 2 μg/mL; Sigma) and F-Hst 5 (30 μM) were added. Time-lapse confocal images were recorded immediately after addition of peptide and PI. Confocal images were acquired with a Zeiss LSM510 Meta Confocal Microscope (Carl Zeiss, Germany) using a Plan Apochromat 63X/1.4 Oil Immersion objective. For *E. faecium*, *S. aureus*, *K. pneumoniae*, and *E. cloacae*, images were collected every 10 min; and for *A. baumannii* and *P. aeruginosa* every 2 min. In order to detect F-Hst 5 and PI simultaneously, the 488 nm line of the argon ion laser and a 561 nm DPSS laser were directed over an HFT UV/488/561 beam splitter, and fluorescence was detected using a Mirror or NFT 565 beam splitter in combination with a BP 500–550 filter for F-Hst 5 and an LP 575 or BP 650–710 filter for PI detection. ImageJ software was used for image acquisition and analysis.

Biofilm Killing Assays

To evaluate killing of bacterial biofilms by Hst 5, cells were inoculated at 1×10^7 cells/ml into 96 well plates and grown overnight at 37°C and 90 RPM for 12 h to form biofilms. After 12 h, spent media was replaced with fresh media, and biofilms were grown for another 4 h in the same conditions. Media was removed and biofilms were incubated with 30 μM Hst 5 suspended in 10 μM NaPB with 10 μM propidium iodide (PI) for 1 h at 37°C, except for *S. aureus* which was incubated at RT. After 1 h, biofilms were gently scraped to remove them from each well, diluted, and placed on microscope slides for image acquisition using a Zeiss Axioobserver.Z1 inverted fluorescent microscope, and an Axiocam 503M camera. Percent killing was calculated as the number of PI positive cells divided by the total number of cells from at least 25 fields from two independent wells.

Hst 5 Killing Activity Determination in Energy Deprived Cells

Overnight cultures of *E. faecium*, *S. aureus*, *A. baumannii*, and *E. cloacae* were diluted and pre-incubated with 10% of media with or without 10 mM NaN_3 at 37°C. *P. aeruginosa* cells were pre-incubated in 10 mM NaPB with 10 mM NaN_3 at 37°C due to altered cell viability in the presence of 10% media with NaN_3. After 3 h incubation, cells were collected by centrifugation and washed two times with NaPB; then cells were used for Hst 5 bactericidal assay in NaPB as described above.

Susceptibility Assay

Minimum inhibitory concentration (MIC) was determined by broth microdilution method based on the guide of Clinical and Laboratory Standards Institute (Clinical Laboratory Standards Institute, 2012) with some modifications. Briefly, all bacterial strains except *K. pneumoniae* were cultured in respective media and grown overnight at 37°C. Cells were washed with NaPB buffer, diluted in 10% of Mueller-Hinton (MH) broth (Sigma) to obtain a concentration of 5×10^6 CFU/mL that were used as the inoculum. Hst 5 peptide was serially diluted in 10% MH broth (since it is inactive in undiluted broth) in 96-well flat-bottomed plates (Falcon). After adding equal volume of target bacterial suspension to the peptide solution, the 96-well plates were incubated at 37°C for 24 h. The MIC values were determined by visibly recording the lowest concentration of Hst 5 that inhibited growth.

Statistics

Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA) using unpaired Student's *t*-tests. Differences of $P < 0.05$ were considered significant. All experiments were performed at least thrice.

RESULTS

Hst 5 Has Strong Bactericidal Activity against Four ESKAPE Pathogens

In order to determine the bactericidal activity of Hst 5 against ESKAPE pathogens, six clinical isolates (**Table 1**) were tested using bactericidal assays (performed in 10 and 100 mM NaPB buffer) to determine percent bacterial killing following incubation for 1 min, 1, and 5 h with 30 μM Hst 5. *E. faecium* showed time-dependent killing since only 18.1 ± 8.9 percent of cells were killed by Hst 5 at 1 h, while killing was enhanced to 63.5 ± 3.5 percent after 5 h incubation (**Figure 1A**). *S. aureus* cells incubated for only 1 min with 30 μM Hst 5 showed 28.3 ± 2.3 percent killing which increased to 60.3 ± 5.7 and 69.7 ± 0.2 percent after 1 and 5 h, respectively (**Figure 1B**). A MRSA strain of *S. aureus* had lower but still significant killing by Hst 5 after 1 h (33.8 ± 3.5%). Interestingly, Hst 5 did not show any killing activity against *K. pneumoniae* for up to 5 h of incubation (**Figure 1C**). *A. baumannii* AB307-0294 had substantial sensitivity to Hst 5 with 31.4 ± 3.7, 90.0 ± 1.1, and 95. ± 2.3 percent killing at 1 min, 1, and 5 h, respectively (**Figure 1D**), while the *A. baumannii HUMC1* strain had even higher sensitivity with 100% killing at 1 h. Similarly, Hst 5 also showed strong killing activity against *P. aeruginosa* 94-323-0635, with a faster rate of killing. At just 1 min of incubation, 83.2 ± 1.3 percent killing was observed; while over 99.9 percent of cells were killed after 1 h treatment (**Figure 1E**). *P. aeruginosa* PAO1 had identical sensitivity to Hst 5 with 100% of cells killed at 1 h. *E. cloacae* sensitivity was similar to that of *A. baumannii* showing 20.3 ± 1.5, 66.1 ± 0.6, and 78.1 ± 1.7 percent of cells killed by Hst 5 at 1 min, 1 h, and 5 h, respectively (**Figure 1F**).

Since the activity of Hst 5 against *C. albicans* is abolished at higher phosphate buffer concentrations (Helmerhorst et al., 1999), Hst 5 killing activity against ESKAPE strains was also tested in a buffer with higher ionic strength. Killing activities of Hst 5 against *E. faecium*, *P. aeruginosa*, and *E. cloacae* were greatly reduced (**Figures 1A,E,F**) when carried out in 100 mM NaPB. However, bactericidal activity against *S. aureus* at 100 mM NaPB was similar to that observed in 10 mM NaPB (**Figure 1B**), while killing efficiency for *A. baumannii* were decreased by about 30 percent in 100 mM NaPB after 1 or 5 h incubation (**Figure 1D**). Effects of PBS on killing were similar to those seen in 100 mM NaPB (data not shown).

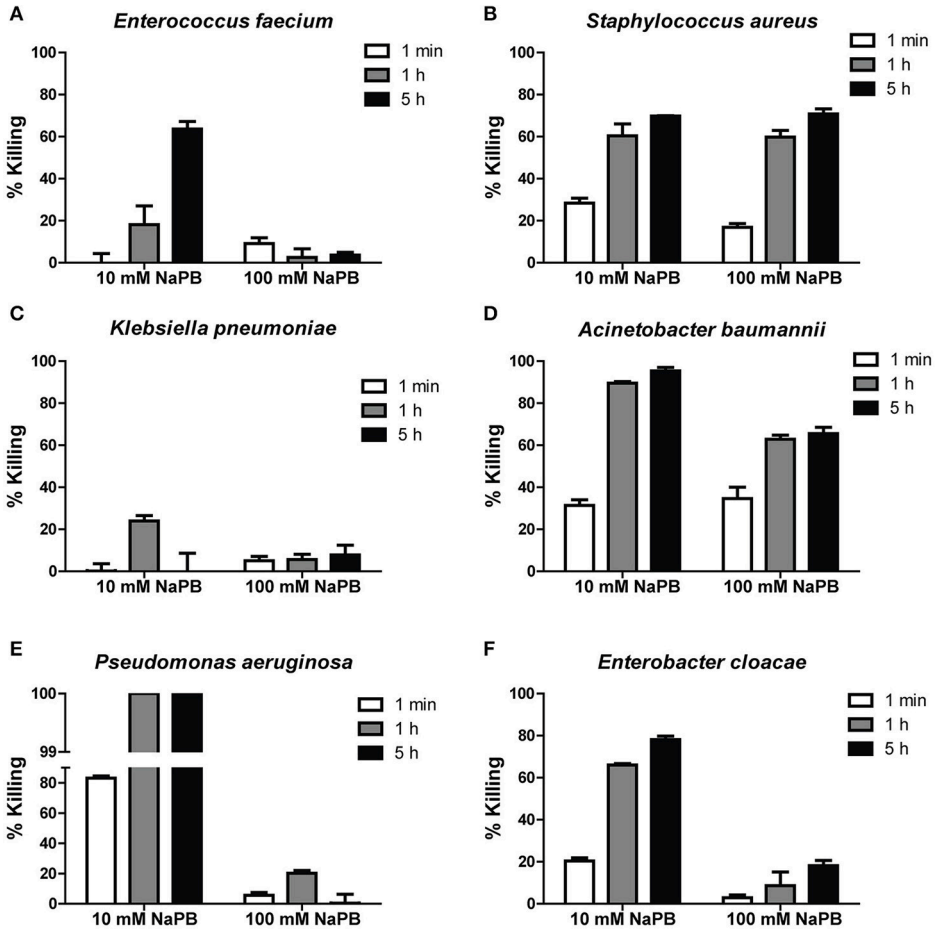


FIGURE 1 | Killing of ESKAPE cells by Hst 5. *E. faecium* **(A)**, *S. aureus* **(B)**, *K. pneumoniae* **(C)**, *A. baumannii* **(D)**, *P. aeruginosa* **(E)**, and *E. cloacae* **(F)** cells in exponential growth were exposed to 30 μM Hst 5 in 10 mM NaPB or 100 mM NaPB for 1 min, 1, and 5 h. Aliquots taken at different time points were diluted and plated. CFU were determined after 24 h. Error bars represent the standard errors from at least three independent replicates of each strain.

Based on these bactericidal results, we next tested growth inhibition by Hst 5 for these ESKAPE pathogens by determining MIC values in 10% MH broth (since Hst 5 is inactive in undiluted broth). Hst 5 MIC values of 38, 47, and 90 μM were observed for *A. baumannii*, *P. aeruginosa,* and *E. cloacae*; while we could not determine MIC values for *E. faecium* and *S. aureus* due to media components that inactivate Hst 5 (**Table 2**).

The activity of Hst 5 against ESKAPE pathogens grown as biofilms was determined after 16 h growth of each bacteria in 96-well plates (**Table 2**). Biofilms were treated with 30 μM Hst 5 for 1 h, dead cells stained with PI, and percent killing calculated. Neither *E. faecium* or *E. cloacae* formed robust biofilms in our hands so that Hst 5 killing could not be reliably determined for these two pathogens. As for planktonic cells, both strains of *P. aeruginosa* in biofilms were effectively killed by Hst 5 after 1 h (59.5%); however Hst 5 killing of *A. baumannii* biofilm cells was reduced to 15.1%; while killing of *S. aureus* biofilm cells (both strains) was negligible (5.1%). Surprisingly, we observed that Hst 5 killed 19.5% of *K. pneumoniae* when in biofilms, although it had no activity against planktonic cells.

Overall, our results show that Hst 5 has a strong bactericidal activity against *P. aeruginosa* and *E. cloacae* (and weaker killing with *E. faecium*) at lower ionic strength environments; while exerting killing against *A. baumannii* and *S. aureus* over a range of ionic strengths. However, biofilm cells of *A. baumannii* and *S. aureus* were more resistant to Hst 5 killing. To confirm these findings in real time and to assess whether ionic strength may influence metabolic or membrane lytic mechanisms of killing, we next measured Hst 5 binding, uptake, and time of killing for these ESKAPE pathogens in 10 mM NaPB.

Bactericidal Activity of Hst 5 against *E. faecium* and *E. cloacae* Requires Internalization and Is Energy Dependent

Time-lapse confocal microscopy showed that Hst 5 (30 μM) rapidly (<1 min) bound with the surface of *E. faecium* cells, although no internalization was evident until after 10 min of incubation (**Figure 2A**). This slow internalization resulted in only 15% of cells taking up F-Hst 5 by 120 min; but in

TABLE 2 | Hst 5-mediated killing of ESKAPE pathogens.

	% Hst 5 killing[a]	% Hst 5 biofilmKilling[b]	% Hst 5-spd killing[c]	Main mechanism of killing		MIC (μM)[d]
				Membrane disruption	Energy dependent	
Enterococcus faecium	63.6	ND	96.6	-	+	ND
Staphylococcus aureus	69.8	3.2	69.2	-	-	ND
Klebsiella pneumoniae	0.0	19.5	0.0	N/A	N/A	N/A
Acinetobacter baumannii	95.34	15.1	96.8	+	-	38
Pseudomonas aeruginosa	>99.9	59.5	>99.9	+	-	47
Enterobacter cloacae	78.1	ND	90.1	-	+	90

[a] *Hst 5 (30 μM) killing efficiency of planktonic cells in 10 mM NaPB buffer for 5 h.*
[b] *Hst 5 (30 μM) killing efficiency of biofilm cells in 10 mM NaPB buffer for 1 h.*
[c] *Hst 5-Spd (30 μM) killing efficiency in 10 mM NaPB buffer for 5 h.*
[d] *MIC in 10% MH broth.*
ND, Not Determined; N/A, Not Applicable.

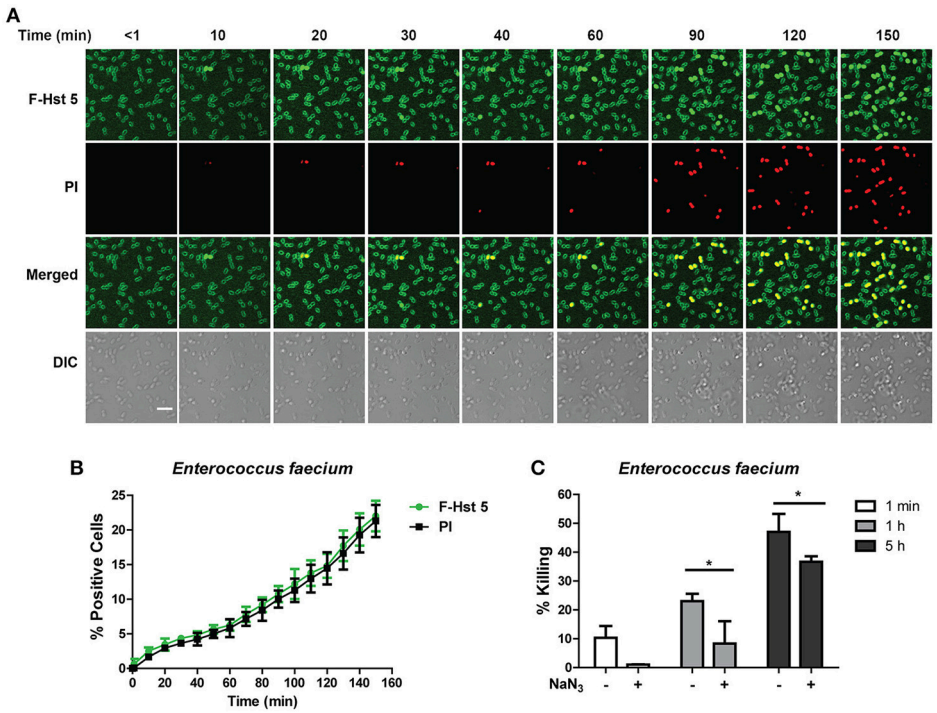


FIGURE 2 | Antibacterial activity of Hst 5 against *E. faecium* required internalization and is energy dependent. (A) *E. faecium* cells were exposed to F-Hst 5 (30 μM) and PI (2 μg/mL). F-Hst 5 (green) and PI (red) uptake were measured in parallel by time-lapse confocal microscopy. Images were recorded every 10 min and selected images of indicated time points were shown. (Scale bar: 5 μm) **(B)** Quantitative analysis of F-Hst 5 uptake (green line) and PI uptake. Error bars represent the standard errors from four different fields of image. **(C)** Cells pretreated with 10 mM NaN_3 at 37°C for 3 h showed decreased susceptibility to Hst 5. (*$P < 0.05$, Student's *t*-test).

each instance, Hst 5 internalization was accompanied by PI uptake (indicative of cell death; **Figure 2B**). This close correlation between intracellular Hst 5 and PI uptake suggested that targets of Hst 5 are intracellular rather than its effects being membrane lytic. However, the percentage of killing from the bactericidal assay was higher than apparent PI staining, suggesting that some portion of killing was delayed. To evaluate this, we pretreated cells with an inhibitor of energy metabolism (NaN_3) as a means to determine whether the killing activity of Hst 5-treated *E. faecium* might be dependent upon cell metabolism. As expected, pretreatment of cells with NaN_3 significantly reduced the killing efficiency of Hst 5 at 1 and 5 h (**Figure 2C**), showing that some portion of Hst 5 killing of *E. faecium* requires target cell energy, likely for Hst 5 internalization or intracellular organelle localization.

E. cloacae cells had a very similar response when treated with F-Hst 5, in that the peptide associated with the surface of all cells almost immediately, however entry of F-Hst 5 (and

accompanying PI staining) was slow and only occurred in 20% of cells within 30 min (**Figure 3A**). As for *E. faecium*, there was a close correlation between intracellular Hst 5 and PI uptake at all time points (**Figure 3B**), also pointing toward intracellular targets for bactericidal activity. *E. cloacae* cells pretreated with 10 mM NaN_3 prior to exposure with 30μM Hst 5 were more resistant to the killing action of F-Hst 5 (**Figure 3C**), similar to the energy dependent mechanism found with *E. faecium*.

Membrane Disruption Is Involved in Bactericidal Activity of Hst 5 against *P. aeruginosa* and *A. baumannii*

In agreement with its high bactericidal activity, F-Hst 5 completely covered the cell surface of *P. aeruginosa* within 1 min, and PI positive cells could be visualized within 2 min (**Figure 4A**). Quantitative analysis showed that 75% of cells contained F-Hst 5 and were PI positive within 5 min of addition of F-Hst 5 (**Figure 4B**). Both intracellular localization of Hst 5 and PI staining were rapid and simultaneous, pointing toward membrane lytic activity of Hst 5 mediated killing of *P. aeruginosa*. Pretreatment of *P. aeruginosa* cells with 10 mM NaN_3 did not reduce the killing activity of Hst 5 (**Figure 4C**), also suggesting energy-independent membrane disruption as the main pathway for Hst 5 killing activity against *P. aeruginosa*.

A. baumannii showed a very similar profile to *P. aeruginosa* after exposure to F-Hst 5 in that F-Hst 5 was associated with the surface of all cells within 2 min; and PI positive cells were visualized with 2 min (**Figure 5A**). Although intracellular uptake of F-Hst 5 was very rapid with 70% of cells containing F-Hst 5 in just 2 min, PI staining did not occur simultaneously so that 70% PI positive cells was seen only after 25 min (**Figure 5B**). Interestingly, the portion of *A. baumannii* cells that showed slower PI staining already contained F-Hst 5; so we questioned whether bactericidal activity of these cells was energy-dependent. However, NaN_3-pretreated *A. baumanni* cells had no difference in F-Hst 5 killing (**Figure 5C**), indicating that Hst 5-mediated killing of *A. baumanni* is partially due to membrane lysis and is energy independent, although some portion of killing might also be a result of its effect on intracellular targets.

Hst 5-Mediated Killing of *S. aureus* Is Delayed and Energy Independent

F-Hst 5 was visualized binding to the cell surface of all *S. aureus* cells within 1 min after addition, however intracellular

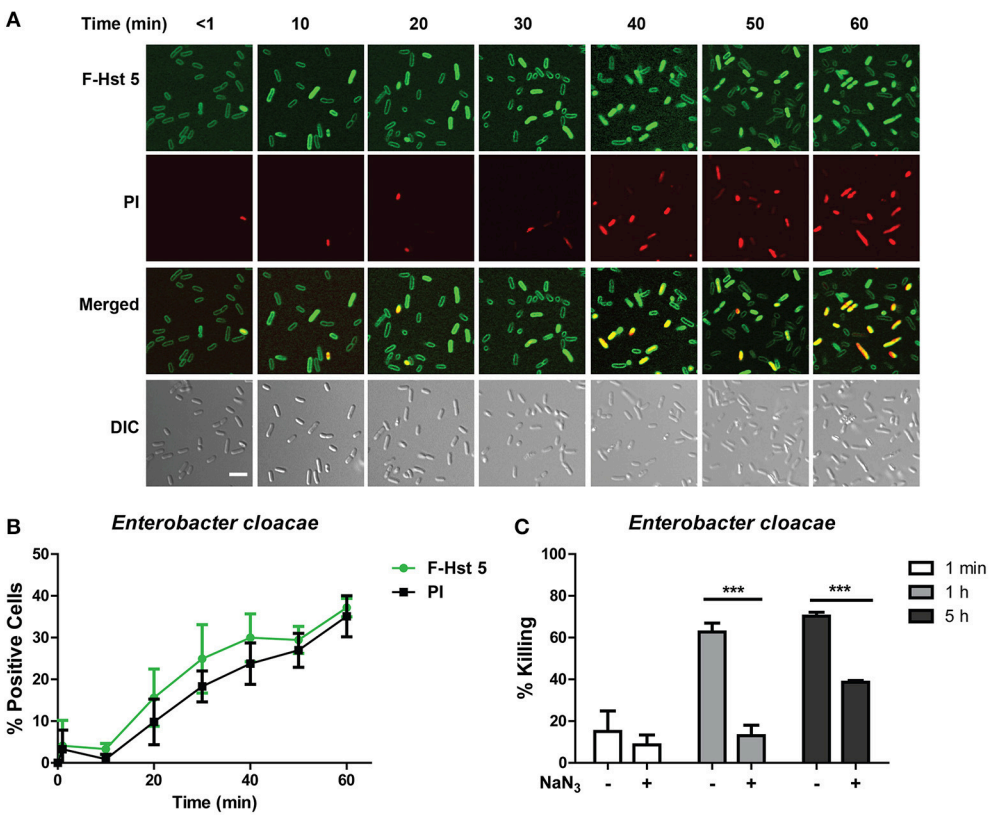


FIGURE 3 | Antibacterial activity of Hst 5 against *E. cloacae* requires internalization and is energy dependent. (A) *E. cloacae* cells were exposed to F-Hst 5 (30 μM) and PI (2 μg/mL). The F-Hst 5 (green) and PI (red) uptake were measured in parallel by time-lapse confocal microscopy. Images were recorded every 10 min and selected images as indicated time points were shown. (Scale bar: 5 μm) **(B)** Quantitative analysis of F-Hst 5 uptake (green line) and PI uptake. Error bars represent the standard errors from four different fields of image. **(C)** Cells pretreated with 10 mM NaN_3 at 37°C for 3 h showed more resistance to Hst 5. (***$P <$ 0.001, Student's *t*-test).

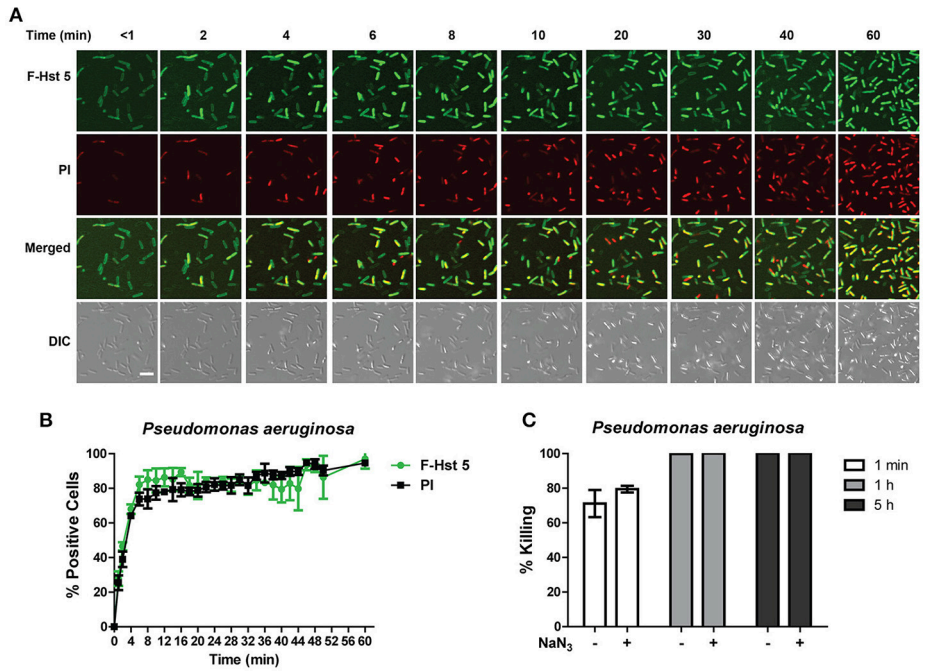


FIGURE 4 | Hst 5 bactericidal activity against *P. aeruginosa* cells is primarily mediated by membrane disruption. (A) *P. aeruginosa* cells were exposed to F-Hst 5 (30 μM) and PI (2 μg/mL). F-Hst 5 (green) and PI (red) uptake were measured in parallel by time-lapse confocal microscopy. Images were recorded every 10 min and selected images at indicated time points were shown (Scale bar: 5 μm) **(B)** Quantitative analysis of F-Hst 5 uptake (green line) and PI uptake. Error bars represent the standard errors from four different fields of image. **(C)** Cells pretreated with 10 mM NaN_3 at 37°C for 3 h did not show significant difference in susceptibility to Hst 5.

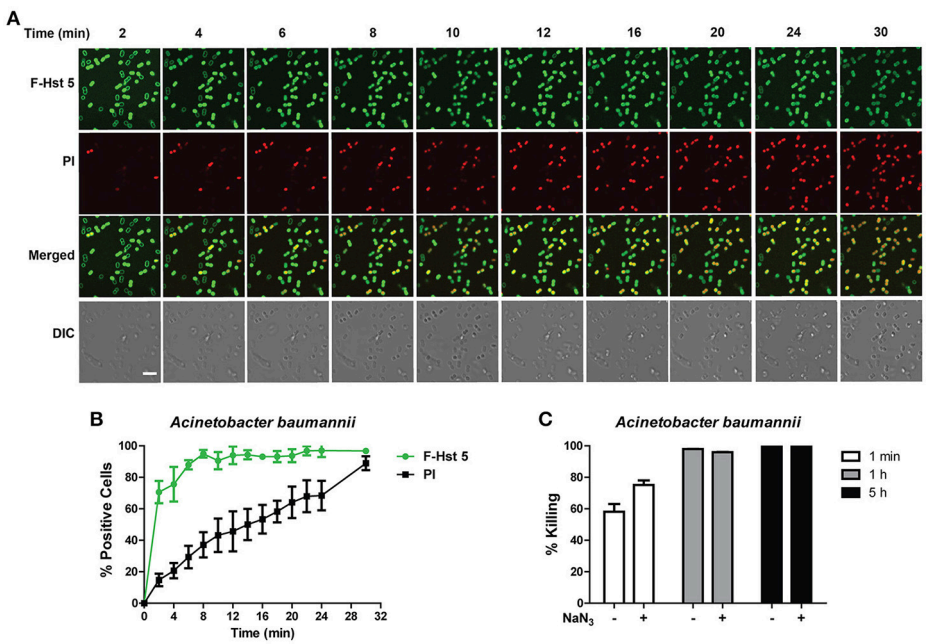


FIGURE 5 | Activity of Hst 5 against *A. baumannii* is mediated in part by membrane disruption. (A) *A. baumannii* cells in exponential phase were exposed to F-Hst 5 (30 μM) and PI (2 μg/mL). The F-Hst 5 (green) and PI (red) uptake were measured in parallel by time-lapse confocal microscopy. Images were recorded every 2 min and selected images as indicated time points were shown. (Arrow, cells of F-Hst 5 uptake positive but without PI uptake; Scale bar: 5 μm). **(B)** Quantitative analysis of F-Hst 5 uptake (green line) and PI uptake. Error bars represent the standard errors from four different fields of image. **(C)** Cells pretreated with 10 mM NaN_3 at 37°C for 3 h did not show significant difference in susceptibility to Hst 5.

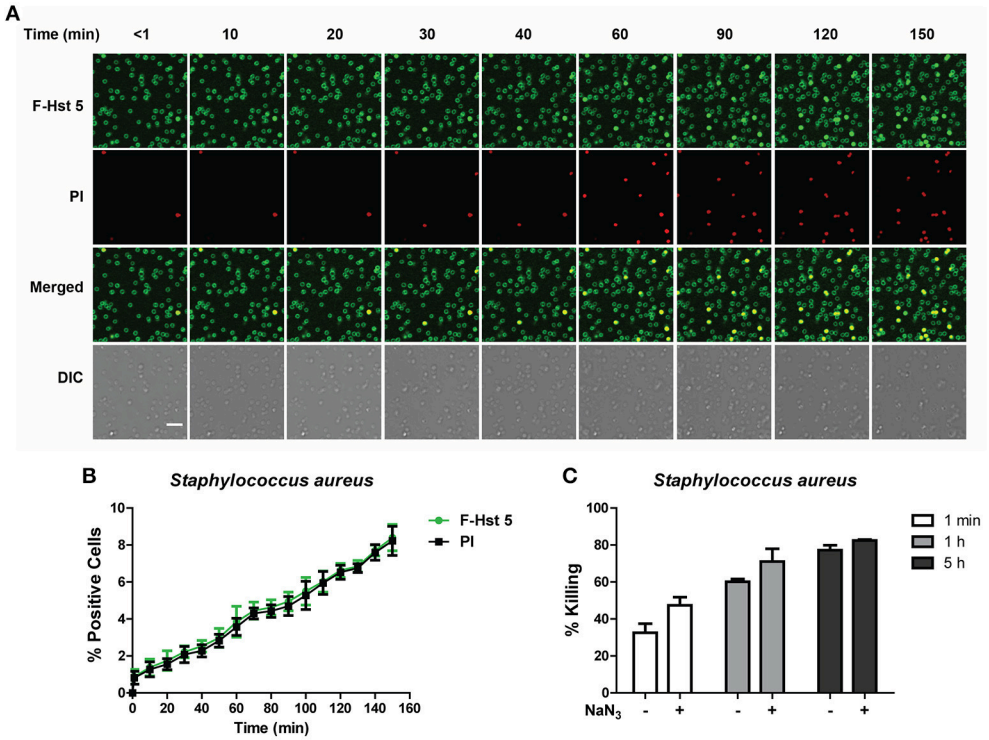


FIGURE 6 | The activity of Hst 5 against *S. aureus* is mediated by energy-independent mechanisms. (A) *S. aureus* cells were exposed to F-Hst 5 (30 μM) and PI (2 μg/mL). F-Hst 5 (green) and PI (red) uptake were measured in parallel by time-lapse confocal microscopy. Images were recorded every 10 min and selected images of indicated time points were shown. (Scale bar: 5 μm). **(B)** Quantitative analysis of F-Hst 5 uptake (green line) and PI uptake. Error bars represent the standard errors from four different fields of image. **(C)** Cells pretreated with 10 mM NaN_3 at 37°C for 3 h did not show significant difference in susceptibility to Hst 5.

localization and PI uptake occurred slowly (**Figure 6A**), so that by 120 min only 7% of cells contained F-Hst 5 and were PI positive (**Figure 6B**). This was surprising since bactericidal activity of Hst 5 after 1 h incubation was found to have 60% killing (**Figure 1**). Furthermore, bactericidal assay with *S. aureus* pretreated with 10 mM NaN_3 did not decrease the killing efficiency (**Figure 6C**). These results, combined with the salt-insensitive killing, suggest that there may be multiple targets for Hst 5-mediated killing of *S. aureus* that are non-lytic and energy independent.

Hst 5 Is Ineffective in Killing *K. Pneumoniae* due to Lack of Sustained Binding

Since Hst 5 showed negligible killing against *K. pneumoniae* (**Figure 1C**), we examined the reason for this using confocal microscopy. F-Hst bound to most *K. pneumoniae* cells within 2 min, however by 10 min most binding was lost suggesting detachment of Hst 5 from the surface capsule (**Figure 7A**). As expected, F-Hst 5 and PI uptake were extremely limited (only 2% of cells), even up to 150 min (**Figure 7B**). These results suggest that F-Hst 5 is released from the *K. pneumoniae* after initial binding (perhaps due to lower binding efficacy in the presence of its capsule) and thus Hst 5 is unable to gain entry or lyse the cells and therefore is ineffective against *K. pneumoniae*.

Hst 5-Spd Has Improved Bactericidal Efficiency against ESKAPE Pathogens That Take Up Hst 5

We have previously reported that spermidine-conjugated Hst 5 (Hst 5-Spd) has greater activity against *C. albicans* because the spermidine conjugate translocates more efficiently into the yeast cells (Tati et al., 2014). Therefore, to determine if Hst 5-Spd has improved bactericidal activity against ESKAPE pathogens as well, we tested the killing activity of Hst 5-Spd [30 μM] against all the six strains (**Figure 8**). Interestingly, Hst 5-Spd had significantly higher killing activity compared with Hst 5 against *E. faecium*, *E. cloacae,* and *A. baumannii* (**Figure 8**). Hst 5-Spd bactericidal effects were most improved against *E. faecium*; increased by three-fold after 1 h incubation and by 1.5-fold after 5 h, while Spermidine (Spd) alone had no killing activity even after 5 h treatment (**Figure 8A**). Hst 5-Spd bactericidal activity was also increased significantly in *E. cloacae* compared with Hst 5, although by only 15% at 1 h and at 5 h. Hst 5-Spd also showed a small but significant increase in killing activity against *A. baumannii* compared with Hst 5 (23% at 1 min and 10% at 1 h). However, after 5 h there was no significant difference perhaps due to the high killing that occurred as a result of Spd (30 μM) itself (**Figure 8D**). These three species of ESKAPE pathogens were also ones that we found to involve Hst 5 uptake and intracellular targets (**Figures 2**, **3**, **5**), suggesting that improved Hst 5-Spd

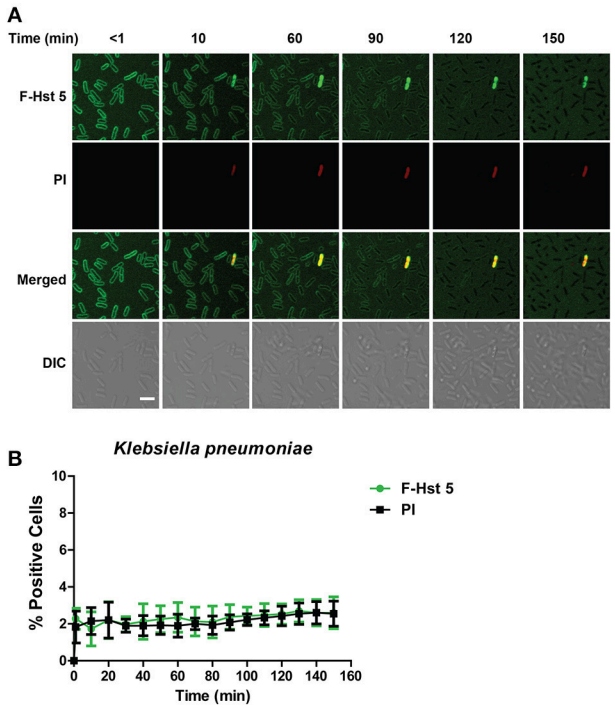


FIGURE 7 | Hst 5 is minimally active against *K. pneumoniae*. (A) *K. pneumoniae* cells in exponential phase were exposed to F-Hst 5 (30 μM) and PI (2 μg/mL). F-Hst 5 (green) and PI (red) uptake were measured in parallel by time-lapse confocal microscopy. Images were recorded every 10 min and selected images of indicated time points were shown. (Scale bar: 5 μm). **(B)** Quantitative analysis of F-Hst 5 uptake (green line) and PI uptake. Error bars represent the standard errors from four different fields of image.

activity in these strains is a result of improved uptake. This is supported by our data showing that the killing ability of Hst 5-Spd against *P. aeruginosa* was not improved, in agreement with a membrane lytic mechanism for this organism. Furthermore, the Hst 5 resistant pathogen *K. pneumoniae* was not affected by Hst 5-Spd showing that this conjugate does not improve binding to this encapsulated ESKAPE pathogen.

DISCUSSION

Here we report the remarkable finding that although Hst 5 activity has been believed to be limited to fungi, Hst 5 also demonstrates very high bactericidal activity against several ESKAPE pathogens. Up to now, a few reports have described interactions between Hst 5 and bacteria. Hst 5 was found to have low killing against *Streptococcus gordonii* (a Gram-positive commensal bacterium of the human oral cavity; Andrian et al., 2012). Zinc-mediated killing of *E. faecalis* bacteria by histidine–rich histatin analogs was found (Rydengard et al., 2006), and killing activity of another histatin derivative (P113) against *P. aeruginosa* and *S. aureus in vitro* was shown (Giacometti et al., 2005). Hst 5 may have other non-bactericidal activities in that it attenuates chemokine responses by binding to *Porphyromonas gingivalis* hemagglutinin B (Borgwardt et al., 2014). Here we expand the scope of Hst 5 activity by showing that full length Hst 5 and an Hst 5 spermidine conjugate both exert very significant killing of all but one ESKAPE pathogen by multiple mechanisms.

The mechanisms by which Hst 5, a naturally occurring salivary protein, kills *C. albicans* have been well-studied and suggest the involvement of multiple intracellular targets (Puri and Edgerton, 2014). The effects on fungal targets range from non-lytic leakage of ATP and K^+ ions to mitochondrial damage and oxidative stress generation (Puri and Edgerton, 2014); and more recently, potential metal scavenging causing nutritional stress (Puri et al., 2015). However, it has been clearly shown that the killing is non-lytic and energy dependent. Interestingly, the ambiguity in the mechanisms of antimicrobial peptides seems universal. Many antibacterial peptides that were considered to be purely lytic are now believed to involve other killing mechanisms ranging from bacterial cell wall disruption to effects on protein and nucleic acid synthesis (Brogden, 2005). We found that Hst 5 can efficiently kill ESKAPE pathogens killing using a unique blend of lysis and energy dependency.

Antibiotic resistance has been a challenge for treating drug resistant bacterial infections and ESKAPE pathogens are no exception (Rice, 2010). One classic drug resistance mechanism entails efflux of the antimicrobial molecules by bacterial cells (Sun et al., 2014). However, Hst 5 resistance is mediated by efflux through *C. albicans* Flu1 transporters (Li et al., 2013) and is not likely to occur in bacteria, as there are no similar transporters known in bacteria. In contrast, *C. albicans* Dur3 transporters (used in polyamine uptake in fungi) are needed for Hst 5 uptake and candidacidal activity (Kumar et al., 2011). Interestingly all ESKAPE pathogens have spermidine/polyamine transporter homologs (Palmer et al., 2010; Ren et al., 2010; Park et al., 2011; Liu et al., 2012; Yao and Lu, 2014; Wang et al., 2016) that might mediate Hst 5 uptake in pathogens that require internalization for killing. Also, the improved bactericidal activity of the Hst 5-spermidine conjugate against *E. faecium* (whose spermidine transporter has the highest homology to the *C. albicans* Dur3 transporter, our unpublished data), and to some extent against *E. cloacae* and *A. baumannii* may be a result of better uptake due to similarity of polyamine transporters expressed in these cells. Biofilm formation is another resistance mechanism that leads to poor drug penetration and accessibility (del Pozo and Patel, 2007; Hoiby et al., 2010). It has previously been shown that Hst 5 is effective against *C. albicans* biofilm (Konopka et al., 2010) and thus Hst 5 can potentially make its way through the polysaccharide extracellular biofilm matrix of ESKAPE bacteria as well.

Prophylactic antimicrobial therapy, especially when applied topically, can be of great advantage to prevent surgical, wound, and burn infections. However, depending on the specificity of the agent used, this may also lead to the killing of healthy flora at the site of application that prevents colonization of pathogens. Here we show that salivary Hst 5 that has limited antibacterial activity against most human oral commensal organisms including *Streptococcal* sp. (found on the human skin; Dale and Fredericks, 2005; Belkaid and Segre, 2014), is extremely effective against *P. aeruginosa* and *A. baumanni*. This provides

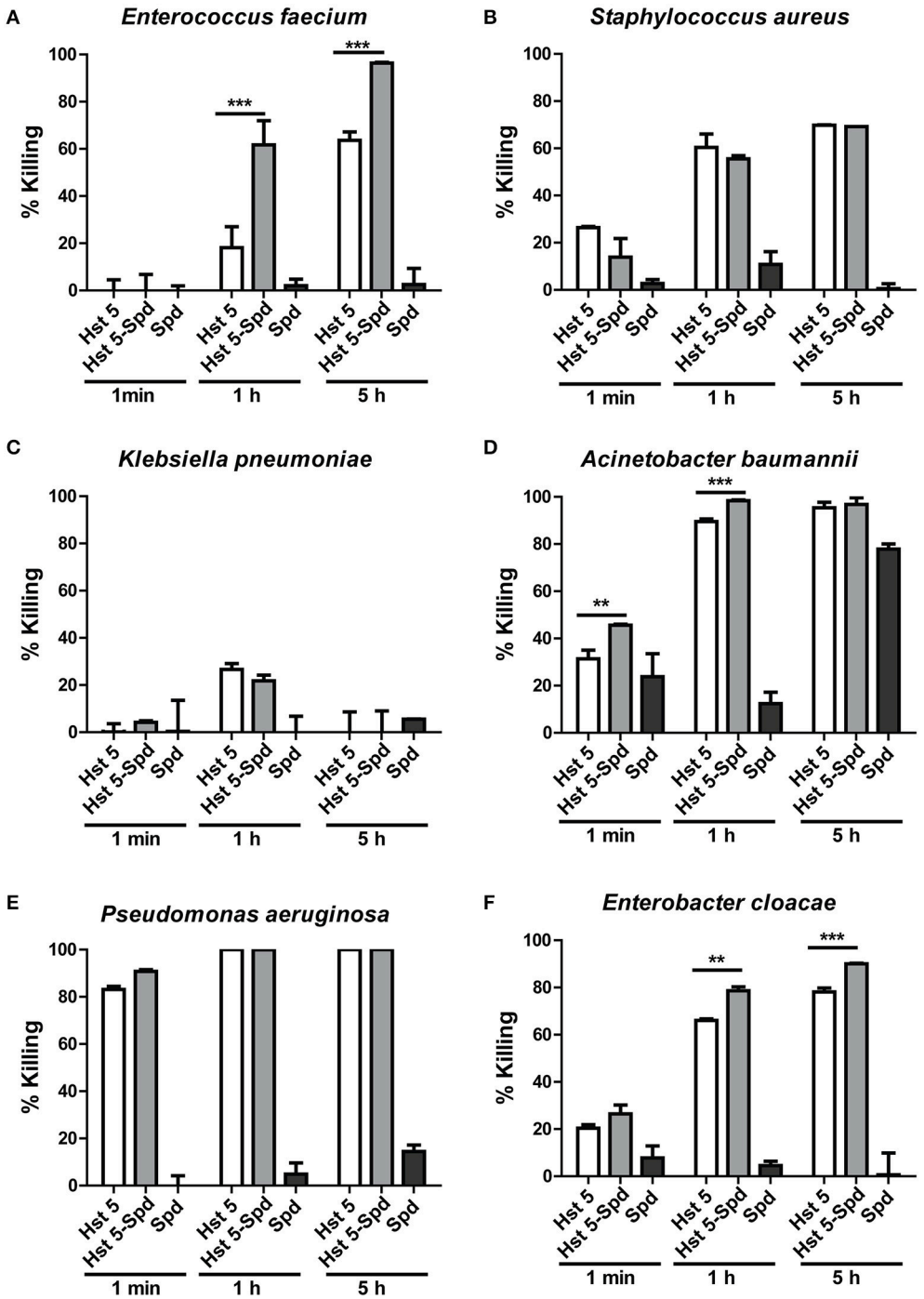


FIGURE 8 | Killing of ESKAPE pathogens by Hst 5-Spd. *E. faecium* **(A)**, *S. aureus* **(B)**, *K. pneumoniae* **(C)**, *A. baumannii* **(D)**, *P. aeruginosa* **(E)**, and *E. cloacae* **(F)** cells in exponential growth were exposed to 30 μM of Hst 5, F-Hst 5 and spermidine in 10 mM NaPB for 1 min, 1, and 5 h. Aliquots taken at different time points were diluted and plated. CFU were determined after 24 h. Error bars represent the standard errors from at least three independent replicates of each strain. Hst 5-Spd conjugate showed more killing efficiency against *E. faecium* **(A)**, *A. baumannii* **(D)**, and *E. cloacae* **(F)** (**$P < 0.01$, ***$P < 0.001$, Student's *t*-test).

a novel potential therapeutic application for Hst 5 since *P. aeruginosa* is one of the most important causative agents for burn infections (Tredget et al., 1992). Furthermore, *P. aeruginosa* is responsible for a majority of bacterial eye infections related to contact lens use (Cope et al., 2016). While Hst 5 is intrinsically absent from human tears, its exogenous application to treat such infections seems plausible, given the high killing activity of Hst 5 against *P. aeruginosa*. Potential therapeutic use of Hst 5 has

some limitations that are inherent to its cationic nature. Since high salt conditions negatively affect Hst 5 microbicidal activity (Helmerhorst et al., 1999; Jang et al., 2010), this may restrict the use of Hst 5 for treatment of systemic disease, although the Hst 5 derivative P113 was found to be effective for systemic use in a rat model of *P. aeruginosa* sepsis (Cirioni et al., 2004). However Hst 5 has a great potential for application topically, both on human skin and in the eye, especially when carried in hypotonic gels and solutions.

Multiple drugs are sometimes required to completely eradicate drug resistant infections. Although further testing of additional strains of ESKAPE pathogens needs to be done, we show here the killing activity of Hst 5 against ESKAPE pathogens, taken together with how Hst 5 can potentially affects multiple intracellular targets, presents the possibility of using this protein in synergy with other existing antibiotics.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: ME, HD, SP. Performed the experiments: HD, AM, HN. Analyzed the data: HD, AM, HN. Prepared the paper: ME, HD, SP, TR.

FUNDING

This work was supported by NIDCR grants DE10641 and DE022720 to ME.

ACKNOWLEDGMENTS

We thank Dr. Wade J. Sigurdson for assistance in confocal microscopy studies performed in the Confocal Microscopy and Flow Cytometry Facility at University at Buffalo.

REFERENCES

Adams, M. D., Goglin, K., Molyneaux, N., Hujer, K. M., Lavender, H., Jamison, J. J., et al. (2008). Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J. Bacteriol.* 190, 8053–8064. doi: 10.1128/JB.00834-08

Andrian, E., Qi, G., Wang, J., Halperin, S. A., and Lee, S. F. (2012). Role of surface proteins SspA and SspB of *Streptococcus gordonii* in innate immunity. *Microbiology* 158(Pt 8), 2099–2106. doi: 10.1099/mic.0.058073-0

Belkaid, Y., and Segre, J. A. (2014). Dialogue between skin microbiota and immunity. *Science* 346, 954–959. doi: 10.1126/science.1260144

Borgwardt, D. S., Martin, A. D., Van Hemert, J. R., Yang, J., Fischer, C. L., Recker, E. N., et al. (2014). Histatin 5 binds to *Porphyromonas gingivalis* hemagglutinin B (HagB) and alters HagB-induced chemokine responses. *Sci. Rep.* 4:3904. doi: 10.1038/srep03904

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., et al. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* 48, 1–12. doi: 10.1086/595011

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* 3, 238–250. doi: 10.1038/nrmicro1098

Cirioni, O., Giacometti, A., Ghiselli, R., Orlando, F., Kamysz, W., D'Amato, G., et al. (2004). Potential therapeutic role of histatin derivative P-113d in experimental rat models of *Pseudomonas aeruginosa* sepsis. *J. Infect. Dis.* 190, 356–364. doi: 10.1086/421913

Clinical and Laboratory Standards Institute (2012). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 9th Edn.* Approved standard M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute.

Coates, R., Moran, J., and Horsburgh, M. J. (2014). Staphylococci: colonizers and pathogens of human skin. *Future Microbiol.* 9, 75–91. doi: 10.2217/fmb.13.145

Cope, J. R., Collier, S. A., Srinivasan, K., Abliz, E., Myers, A., Millin, C. J., et al. (2016). Contact lens-related corneal infections - United States, 2005-2015. *MMWR Morb. Mortal. Wkly. Rep.* 65, 817–820. doi: 10.15585/mmwr.mm6532a2

Dale, B. A., and Fredericks, L. P. (2005). Antimicrobial peptides in the oral environment: expression and function in health and disease. *Curr. Issues Mol. Biol.* 7, 119–133. doi: 10.1093/jac/dki103

del Pozo, J. L., and Patel, R. (2007). The challenge of treating biofilm-associated bacterial infections. *Clin. Pharmacol. Ther.* 82, 204–209. doi: 10.1038/sj.clpt.6100247

Devine, D. A., and Hancock, R. E. (2002). Cationic peptides: distribution and mechanisms of resistance. *Curr. Pharm. Des.* 8, 703–714. doi: 10.2174/1381612023395501

Giacometti, A., Cirioni, O., Kamysz, W., D'Amato, G., Silvestri, C., Del Prete, M. S., et al. (2005). *In vitro* activity of the histatin derivative P-113 against multidrug-resistant pathogens responsible for pneumonia in immunocompromised patients. *Antimicrob. Agents Chemother.* 49, 1249–1252. doi: 10.1128/AAC.49.3.1249-1252.2005

Groenink, J., Ruissen, A. L., Lowies, D., van't Hof, W., Veerman, E. C., and Nieuw Amerongen, A. V. (2003). Degradation of antimicrobial histatin-variant peptides in *Staphylococcus aureus* and *Streptococcus mutans*. *J. Dent. Res.* 82, 753–757. doi: 10.1177/154405910308200918

Helmerhorst, E. J., Breeuwer, P., van't Hof, W., Walgreen-Weterings, E., Oomen, L. C., Veerman, E. C., et al. (1999). The cellular target of histatin 5 on *Candida albicans* is the energized mitochondrion. *J. Biol. Chem.* 274, 7286–7291. doi: 10.1074/jbc.274.11.7286

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* 35, 322–332. doi: 10.1016/j.ijantimicag.2009.12.011

Houang, E. T., Sormunen, R. T., Lai, L., Chan, C. Y., and Leong, A. S. (1998). Effect of desiccation on the ultrastructural appearances of *Acinetobacter baumannii* and *Acinetobacter lwoffii*. *J. Clin. Pathol.* 51, 786–788. doi: 10.1136/jcp.51.10.786

Jang, W. S., Bajwa, J. S., Sun, J. N., and Edgerton, M. (2010). Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in *Candida albicans*. *Mol. Microbiol.* 77, 354–370. doi: 10.1111/j.1365-2958.2010.07210.x

Keller, R., Pedroso, M. Z., Ritchmann, R., and Silva, R. M. (1998). Occurrence of virulence-associated properties in *Enterobacter cloacae*. *Infect. Immun.* 66, 645–649.

Khan, S. A., Fidel, P. L. Jr., Thunayyan, A. A., Varlotta, S., Meiller, T. F., and Jabra-Rizk, M. A. (2013). Impaired histatin-5 levels and salivary antimicrobial activity against *C. albicans* in HIV infected individuals. *J. AIDS Clin. Res.* 4. doi: 10.4172/2155-6113.1000193

Konopka, K., Dorocka-Bobkowska, B., Gebremedhin, S., and Duzgunes, N. (2010). Susceptibility of Candida biofilms to histatin 5 and fluconazole. *Antonie Van Leeuwenhoek* 97, 413–417. doi: 10.1007/s10482-010-9417-5

Kumar, R., Chadha, S., Saraswat, D., Bajwa, J. S., Li, R. A., Conti, H. R., et al. (2011). Histatin 5 uptake by *Candida albicans* utilizes polyamine transporters Dur3 and Dur31 proteins. *J. Biol. Chem.* 286, 43748–43758. doi: 10.1074/jbc.M111.311175

Li, R., Kumar, R., Tati, S., Puri, S., and Edgerton, M. (2013). *Candida albicans* flu1-mediated efflux of salivary histatin 5 reduces its cytosolic concentration and fungicidal activity. *Antimicrob. Agents Chemother.* 57, 1832–1839. doi: 10.1128/AAC.02295-12

Lindsay, A. K., and Hogan, D. A. (2014). *Candida albicans*: molecular interactions with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Fungal Biol. Rev.* 28, 85–96. doi: 10.1016/j.fbr.2014.10.002

Liu, P., Li, P., Jiang, X., Bi, D., Xie, Y., Tai, C., et al. (2012). Complete genome sequence of *Klebsiella pneumoniae* subsp. pneumoniae HS11286, a multidrug-resistant strain isolated from human sputum. *J. Bacteriol.* 194, 1841–1842. doi: 10.1128/JB.00043-12

Lopes, A. E. R., Canini, S. R. M. D., Reinato, L. A. F., Lopes, L. P., and Gir, E. (2015). Prevalence of gram-positive bacteria in patients with HIV in specialized services. *Acta Paulista De Enfermagem* 28, 281–286. doi: 10.1590/1982-0194201500047

Luo, G., Lin, L., Ibrahim, A. S., Baquir, B., Pantapalangkoor, P., Bonomo, R. A., et al. (2012). Active and passive immunization protects against lethal, extreme drug resistant-Acinetobacter baumannii infection. *PLoS ONE* 7:e29446. doi: 10.1371/journal.pone.0029446

Magill, S. S., Edwards, J. R., Bamberg, W., Beldavs, Z. G., Dumyati, G., Kainer, M. A., et al. (2014). Multistate point-prevalence survey of health care-associated infections. *N. Engl. J. Med.* 370, 1198–1208. doi: 10.1056/NEJMoa1306801

Palmer, K. L., Carniol, K., Manson, J. M., Heiman, D., Shea, T., Young, S., et al. (2010). High-quality draft genome sequences of 28 *Enterococcus* sp. isolates. *J. Bacteriol.* 192, 2469–2470. doi: 10.1128/JB.00153-10

Park, J. Y., Kim, S., Kim, S. M., Cha, S. H., Lim, S. K., and Kim, J. (2011). Complete genome sequence of multidrug-resistant *Acinetobacter baumannii* strain 1656-2, which forms sturdy biofilm. *J. Bacteriol.* 193, 6393–6394. doi: 10.1128/JB.06109-11

Podschun, R., and Ullmann, U. (1998). *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin. Microbiol. Rev.* 11, 589–603.

Puri, S., and Edgerton, M. (2014). How does it kill?: understanding the candidacidal mechanism of salivary histatin 5. *Eukaryotic Cell* 13, 958–964. doi: 10.1128/EC.00095-14

Puri, S., Li, R., Ruszaj, D., Tati, S., and Edgerton, M. (2015). Iron binding modulates candidacidal properties of salivary histatin 5. *J. Dent. Res.* 94, 201–208. doi: 10.1177/0022034514556709

Ren, Y., Ren, Y., Zhou, Z., Guo, X., Li, Y., Feng, L., et al. (2010). Complete genome sequence of *Enterobacter cloacae* subsp. cloacae type strain ATCC 13047. *J. Bacteriol.* 192, 2463–2464. doi: 10.1128/JB.00067-10

Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J. Infect. Dis.* 197, 1079–1081. doi: 10.1086/533452

Rice, L. B. (2010). Progress and challenges in implementing the research on ESKAPE pathogens. *Infect. Control Hosp. Epidemiol.* 31, S7–S10. doi: 10.1086/655995

Rydengard, V., Andersson Nordahl, E., and Schmidtchen, A. (2006). Zinc potentiates the antibacterial effects of histidine-rich peptides against *Enterococcus faecalis*. *FEBS J.* 273, 2399–2406. doi: 10.1111/j.1742-4658.2006.05246.x

Salvatori, O., Puri, S., Tati, S., and Edgerton, M. (2016). Innate immunity and saliva in *Candida albicans*-mediated oral diseases. *J. Dent. Res.* 95, 365–371. doi: 10.1177/0022034515625222

Sands, K. M., Wilson, M. J., Lewis, M. A., Wise, M. P., Palmer, N., Hayes, A. J., et al. (2016). Respiratory pathogen colonization of dental plaque, the lower airways, and endotracheal tube biofilms during mechanical ventilation. *J. Crit. Care* 37, 30–37. doi: 10.1016/j.jcrc.2016.07.019

Sun, J., Deng, Z., and Yan, A. (2014). Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochem. Biophys. Res. Commun.* 453, 254–267. doi: 10.1016/j.bbrc.2014.05.090

Tati, S., Li, R., Puri, S., Kumar, R., Davidow, P., and Edgerton, M. (2014). Histatin 5-spermidine conjugates have enhanced fungicidal activity and efficacy as a topical therapeutic for oral candidiasis. *Antimicrob. Agents Chemother.* 58, 756–766. doi: 10.1128/AAC.01851-13

Tredget, E. E., Shankowsky, H. A., Joffe, A. M., Inkson, T. I., Volpel, K., Paranchych, W., et al. (1992). Epidemiology of infections with *Pseudomonas aeruginosa* in burn patients: the role of hydrotherapy. *Clin. Infect. Dis.* 15, 941–949. doi: 10.1093/clind/15.6.941

Vu, J., and Carvalho, J. (2011). Enterococcus: review of its physiology, pathogenesis, diseases and the challenges it poses for clinical microbiology. *Front. Biol.* 6:357. doi: 10.1007/s11515-011-1167-x

Wang, C., Liu, X., Wang, J., Zhou, J., Cui, Z., and Zhang, L. H. (2016). Design and characterization of a polyamine derivative inhibiting the expression of type III secretion system in *Pseudomonas aeruginosa*. *Sci. Rep.* 6:30949. doi: 10.1038/srep30949

Yao, X., and Lu, C. D. (2014). Functional characterization of the potRABCD operon for spermine and spermidine uptake and regulation in *Staphylococcus aureus*. *Curr. Microbiol.* 69, 75–81. doi: 10.1007/s00284-014-0556-1

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on