ANTIMICROBIAL RESISTANCE AND VIRULENCE COMMON MECHANISMS

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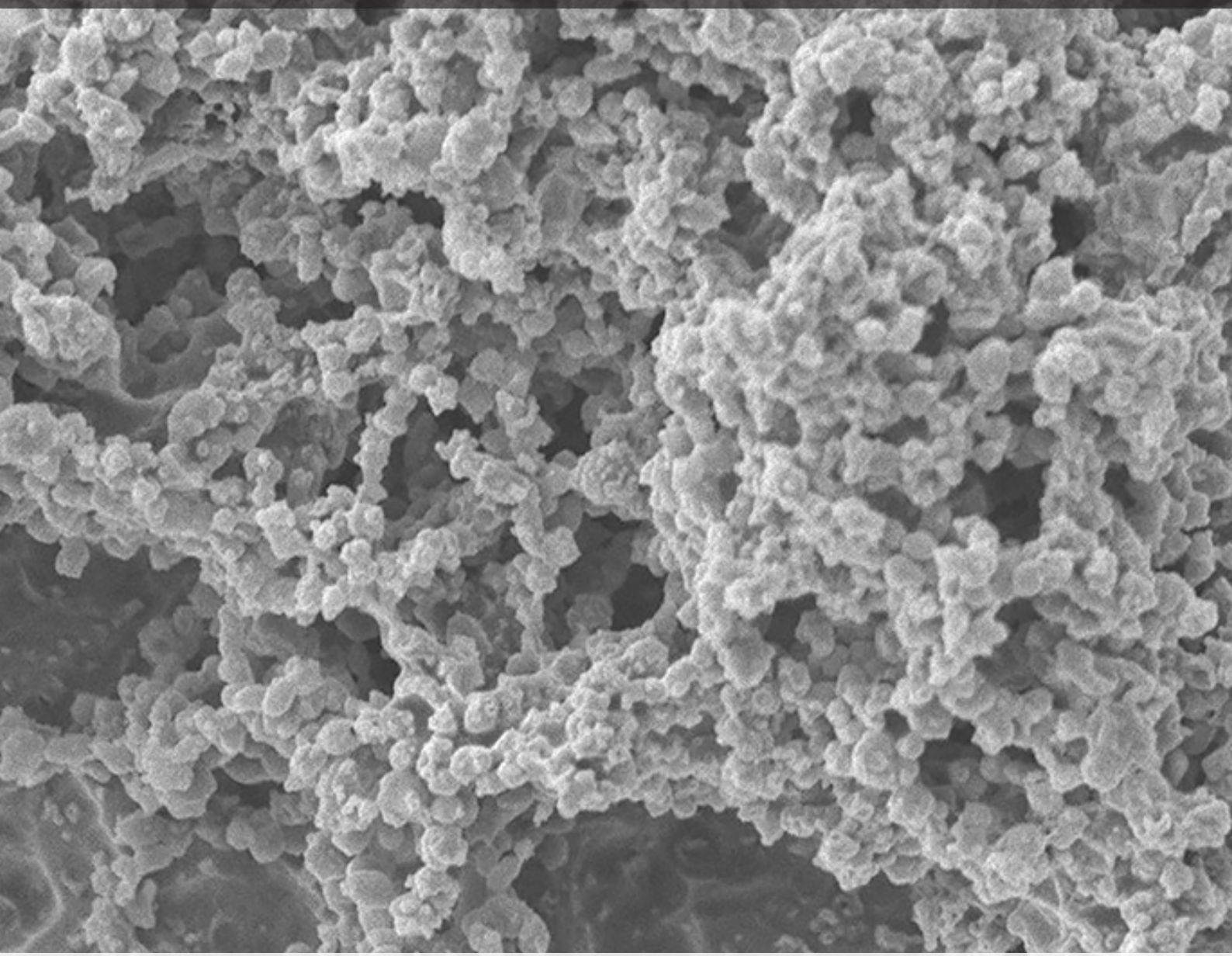

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ANTIMICROBIAL RESISTANCE AND VIRULENCE COMMON MECHANISMS

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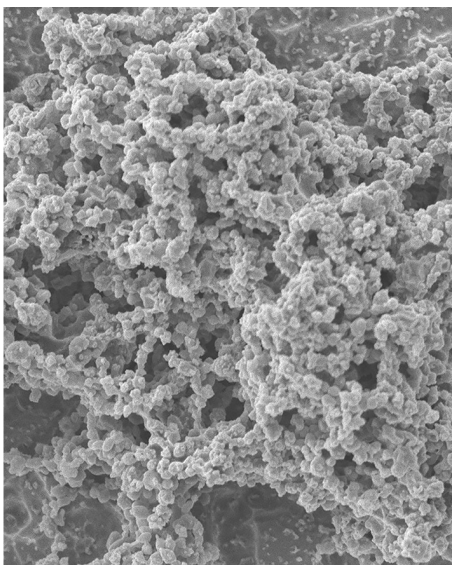

Scanning electron microscopy image of a *Streptococcus suis* biofilm. Certain physiological adaptations like biofilm formation may affect both virulence by increasing resistance to host defense response and resistance to antibiotics by decreasing their access to biofilm embedded bacteria.

Image taken from: Wang S, Yang Y, Zhao Y, Zhao H, Bai J, Chen J, Zhou Y, Wang C and Li Y (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

Multiple relationships exist between antimicrobial resistance and bacterial virulence, and the spread of clones combining multiple antibiotic resistance and a high virulence level is an increasing problem. It was previously described how mutation-driven or horizontally acquired resistance mechanisms can also have effects on virulence. It was also reported that mobile genetic elements often carry both resistance determinants and virulence-modulating genes, which favors the co-selection of both traits. In the present volume, we present a collection of articles which document additional aspects of the interactions between antimicrobial resistance and virulence in bacteria, and describe their potential therapeutic consequences.

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EDITORIAL
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Editorial: Antimicrobial Resistance and Virulence Common Mechanisms

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Editorial on the Research Topic

Antimicrobial Resistance and Virulence Common Mechanisms

Multiple relationships exist between antimicrobial resistance and bacterial virulence, and the spread of clones combining multiple antibiotic resistance and a high virulence level is an increasing problem. These relationships have been previously reviewed, notably by Beceiro et al. (2013), who described how mutation-driven or horizontally acquired resistance mechanisms can also have effects on virulence. It was also reported that mobile genetic elements often carry both resistance determinants and virulence-modulating genes, which favors the co-selection of both traits. In the present volume, we present a collection of articles which document additional aspects of the interactions between antimicrobial resistance and virulence in bacteria, and describe their potential therapeutic consequences.

In an excellent review article, Alcade-Rico et al. established that multidrug efflux pumps are "at the cross-road between resistance and virulence of bacterial pathogens." They describe how multidrug efflux pumps are involved, besides antibiotic resistance, in many physiological and virulence-related processes controlled by complex regulatory networks. A special focus is made on the role of these efflux pumps in bacterial cell-to-cell communication and in bacterial-host interactions.

Another example of link between resistance and virulence in *Staphylococci* is given in a mini review by Qin et al. The authors summarized how the *psm-mec* locus present in some methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative *Staphylococci* influences methicillin resistance and virulence-related phenotypes, including the ability to form biofilms.

In another paper also dealing with biofilms, Wang et al. used an up-to-date iTRAQ protein labeling technology to identify proteins that are differencially expressed in *Streptococcus suis* treated with sub-MIC concentrations of tylosin. These concentrations were indeed capable of inhibition of biofilm formation by this zoonotic pathogen, which is of interest, since biofilms are known to display an increased tolerance to antibiotics.

This antibiotic tolerance of bacteria present in biofilms is thought to be at least partially due to the presence of higher numbers of persisters than in planktonic bacterial populations. Persisters are phenotypic variants of bacteria which are able to survive antibiotic treatments in a dormancy state and to resume growth when the (antibiotic) stress is withdrawn. Like antibiotic-resistant bacteria, antibiotic-tolerant persisters are also often responsible for failures of antibiotic therapies. However, the mechanisms underlying their production are still not well-understood. An article by Xu et al. lifts a corner of the veil on this subject in *Staphylococcus aureus* by suggesting that heme biosynthesis, in addition to being necessary to a full expression of virulence, is also important in the formation of persisters in *S. aureus*.

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Another study by Chen et al. addressed the problem of antibiotic tolerance in extraintestinal pathogenic *Escherichia coli* (ExPEC). Authors studied the role of polyphosphate kinase (PPK), an enzyme already known for its implication in motility, quorum sensing or virulence, in antibiotic tolerance. Their results indicate that PPK is important for the antibiotic stress response and may therefore be a valuable antibacterial target, especially since it is absent in mammals.

Small Colony Variants (SCV) represent another state in which bacteria are more tolerant to antibiotics. The clinical importance of SCV has been well-documented in cases of chronic infections by *S. aureus* and *Pseudomonas aeruginosa* but their existence was only recently reported in *Listeria monocytogenes*, a food pathogen producing recurrent infections despite being rarely resistant to antibiotics. In an original research article, Curtis et al. described a heme mutant displaying a SCV phenotype mutant with an increased tolerance toward most of the relevant antibiotics. They suggest that the SCV phenotype of *L. monocytogenes* should be screened in clinical laboratories, as it might be associated to complicated antibiotic treatments.

Genetic changes leading to antimicrobial resistance are expected to result in higher fitness costs in the absence of antibiotic selection pressure. On the other hand, increased resistance is sometimes associated with increased virulence or to a better adaptation to stress conditions. This topic is developed in a study by Schaufler et al. who reported that the carriage of extended spectrum beta-lactamase (ESBL)-plasmids by strains of pandemic *E. coli* lineages did not lead to a fitness cost. Instead, these plasmids could enhance virulence or adaptation to specific habitats by influencing the expression of chromosomal genes.

In another article dealing with bacterial fitness, Agnello et al. presented results that possibly explain why highly virulent *P. aeruginosa* strains, which often possess the *exoU* toxin gene, are more frequently resistant to fluoroquinolones than the generally less virulent strains harboring the *exoS* toxin gene. Their results support the hypothesis that fitness cost imposed by fluoroquinolone resistance may be lower and "more easily" compensable by *exoU* strains than by *exoS* strains.

In a whole genome sequence-based study, Hao et al. analyze a multidrug resistant and virulent *Campylobacter jejuni* strain isolated from a broiler chicken. By comparison to reference strains, they revealed that the phenotype of this isolate was associated with large differences in the genome structure and content. This study provides an example of how a stepwise accumulation of mutations in antibiotic target genes, acquisition of exogenous resistance genes and virulence associated genes can result in the emergence of isolates that are both multidrug-resistant and virulent.

Since quorum sensing is a major player in the control of virulence factors in *P. aeruginosa*, therapies based on quorum sensing interference (QSI) have been envisaged as possible alternatives to conventional antibiotic therapies against this pathogen. In a thoughtful perspective article, Garcia-Contreras, however, compiles many recent experimental evidence which actually indicate that early optimistic expectations about QSI should be put in perspective. Indeed, he estimates that a far better understanding of *P. aeruginosa* virulence and behavior during infection is needed before efficient QSI-based clinical applications can be designed.

In addition to some immunomodulating activities, azithromycin (AZM) has various inhibiting effects on *P. aeruginosa* such as bacterial killing or the repression of multiple virulence factors. This antibiotic is therefore currently used in the treatment of cystic fibrosis patients, whose lungs are commonly colonized by this pathogen. However, *P. aeruginosa* responds to AZM by yet unknown mechanisms and is able to counteract its killing and virulence-inhibitory effects. An interesting work by Tan et al. revealed that PA3297, a *P. aeruginosa* gene encoding a DEAH-box helicase, is involved in this response. Indeed, deficiency of this gene renders *P. aeruginosa* more susceptible to the killing and to virulence suppression by AZM. Authors therefore suggested that targeting the regulatory pathway of PA3297 or its function might increase the beneficial effect of AZM in chronic *P. aeruginosa* infections such as those occuring in cystic fibrosis patients.

At last, in a pharmacological perspective, Obtreska-Machaj et al. report the development of hydantoin derivatives that have increased activity as inhibitors of the AcrAB-TolC efflux pump of *Enterobacter aerogenes*. Close homologs of this multidrug pump are also present in other pathogenic enterobacteria and involved in their virulence. The authors defined important pharmacophoric groups whose modulation may allow to develop still more potent inhibitors capable of restoring antibiotic activity in AcrAB producing bacteria.

It is likely that both resistant and virulent high risk clones will continue to emerge and spread in the next years. Several factors supports this hypothesis, among which the facts that highly pathogenic bacterial strains are more likely to be subjected to the selective pressure of antibiotics treatments, that virulence and resistance determinants are sometimes genetically associated and that fitness and virulence impairments that often accompany acquisition of resistance can be compensated in the long term by suppressor mutations (Beceiro et al., 2013; Mathers et al., 2015). In spite of these facts, virulence and antimicrobial resistance are studied usually separately rather than jointly. But now, as exemplified in this research topic, time has come for more integrative studies linking both traits and their co-evolution, so that we may be better armed against future bacterial threats.

AUTHOR CONTRIBUTIONS

All authors have substantially contributed to this work and approved it for publication.

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REVIEW
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Multidrug Efflux Pumps at the Crossroad between Antibiotic Resistance and Bacterial Virulence

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Multidrug efflux pumps can be involved in bacterial resistance to antibiotics at different levels. Some efflux pumps are constitutively expressed at low levels and contribute to intrinsic resistance. In addition, their overexpression may allow higher levels of resistance. This overexpression can be transient, in the presence of an effector (phenotypic resistance), or constitutive when mutants in the regulatory elements of the expression of efflux pumps are selected (acquired resistance). Efflux pumps are present in all cells, from human to bacteria and are highly conserved, which indicates that they are ancient elements in the evolution of different organisms. Consequently, it has been suggested that, besides antibiotic resistance, bacterial multidrug efflux pumps would likely contribute to other relevant processes of the microbial physiology. In the current article, we discuss some specific examples of the role that efflux pumps may have in the bacterial virulence of animals' and plants' pathogens, including the processes of intercellular communication. Based in these evidences, we propose that efflux pumps are at the crossroad between resistance and virulence of bacterial pathogens. Consequently, the comprehensive study of multidrug efflux pumps requires addressing these functions, which are of relevance for the bacterial–host interactions during infection.

Keywords: multidrug efflux pumps, quorum sensing, antibiotic resistance mechanisms, virulence, global regulation

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INTRODUCTION

Multidrug resistance (MDR) efflux pumps are relevant elements belonging to the microbial repertoire that bacteria harbor for resisting the action of antimicrobial drugs (Piddock, 2006a; Vila and Martínez, 2008; Li et al., 2015; Jang, 2016). Indeed, several works have shown that these elements are involved in resistance of *in vitro* selected mutants as well as in the reduced susceptibility to antimicrobials of clinical isolates of different bacterial pathogens. The expression of efflux pumps is usually down regulated; only some of them present a substantial level of expression under regular growing conditions in the laboratory (Grkovic et al., 2001, 2002). However, constitutive high-level expression of these elements can be achieved by means of mutations in the elements regulating their expression. Transient high-level expression of efflux pumps can also be triggered in the presence of their effectors or under some specific growing conditions. In agreement with this situation, efflux pumps contribute to antibiotic resistance at three different levels: they can be involved in intrinsic resistance when presenting a basal level of expression under any condition.

They can contribute to acquired resistance when mutants achieving high-level of expression of the efflux pumps are selected. Finally, they can contribute to transient, non-inheritable, phenotypic resistance when bacteria are growing in the presence of an effector of the efflux pump or under growing conditions that trigger their overexpression. As reviewed in Hernando-Amado et al. (2016), efflux pumps are grouped in five structural families, namely the resistance-nodulation-division (RND), the small multidrug resistance (SMR), the multi antimicrobial extrusion (MATE), the major facilitator superfamily (MFS), and the ATP-binding cassette (ABC) superfamilies. Whereas some efflux pumps can work independently of any other protein, mainly in the case of Gram-positive organisms, in the case of Gram-negative organisms, they form tripartite complexes capable to traverse both bacterial membranes. These complexes include the inner-membrane efflux pump, a membrane fusion protein and an outer membrane protein.

When compared with other classical resistance genes, MDR efflux pumps present some specific features that support they should have other roles in the bacterial physiology besides their well-known involvement in antibiotic resistance. First, MDR efflux pumps are ubiquitous; they are present in all living cells, from humans to bacteria (Alonso et al., 1999; Alonso and Martinez, 2001; Gould et al., 2004; Sanchez et al., 2004). Second, the genes encoding them belong to the bacterial core genome in the sense that all (or most) members of a given species harbor the same efflux pumps (Alonso et al., 1999). Third, they are redundant; a single bacterial cell usually contains more than 10 different efflux pumps (Crossman et al., 2008). Fourth, they are rather unspecific; each efflux pump is able to extrude a variety of different substrates, including synthetic antibiotics as quinolones (Hernandez et al., 2011; Redgrave et al., 2014). Fifth, as above mentioned the expression of efflux pumps is tightly regulated; this regulation includes local regulators usually encoded upstream the structural genes of the operon encoding the efflux pump, as well as global regulators (Randall and Woodward, 2002; Luong et al., 2003; Nikaido et al., 2008; De Majumdar et al., 2013), frequently controlling the expression of a set of genes involved in the adaptation to a given ecosystem, as is the infected host. Sixth, at least in occasions, antibiotics are not good effectors of the expression of efflux pumps, whereas host-produced compounds as bile salts or plant-produced signals may induce the expression of MDR pumps (Rosenberg et al., 2003; Prouty et al., 2004; García-León et al., 2014). Altogether, these characteristics support that MDR efflux pumps are ancient elements (present in all organisms), important for the bacterial physiology (all members of a given species present the same, conserved efflux pumps), likely displaying different functions besides antibiotic resistance (a single microorganism contains a large number of different efflux pumps, with overlapping substrate ranges, including synthetic antibiotics not present in nature) and frequently integrated in complex response networks (they form part of global regulons and their expression is triggered by host produced compounds). In the present article we discuss some examples of the potential functions, besides antibiotic resistance, of MDR efflux pumps with a particular focus on the role that they may have in bacterial–host interactions in animals (humans) and plants as well as in intercellular signaling (Piddock, 2006b; Martinez et al., 2009; Alvarez-Ortega et al., 2013).

EFFLUX PUMPS AND CELL-TO-CELL COMMUNICATION

The capability to sense the environment and the organisms that are living in the same niche is critical to allow the microorganisms for choosing the best strategy to survive and colonize such niche. Along evolution, a battery of different mechanisms to sense the continuously changing environment has been selected in different microbial species. One of these mechanisms consists on the cell-to-cell communication systems. These inter-cellular signaling systems are based on the production of one or more low-molecular weight compounds which are sensed by molecular receptors of other cells, promoting a specific response in the target organism. In the bacterial world, this phenomenon is known as quorum sensing (QS) because it was initially described as a mechanism to sense the density of the bacterial population belonging to the same species present in a given habitat. The QS system allows the establishment of a cooperative genetic program of the whole population that increases the microbial efficacy for colonizing a given environment, including the infected host (Williams et al., 2007). The QS signal molecules (QSSMs) are constitutively produced at low quantities by all cells in the population. Their release outside the cells allows a progressive accumulation of QS signals in the intercellular space. The consequence is an increasingly production and accumulation of QSSMs while the population size increases, activating the QS response when the concentration of the signal reaches a threshold level. Further, these molecules (known as autoinducers) are able to induce their own production when they bind to their cognate transcriptional regulators, which produce a feed-forward regulation circuit. The regulator-QSSM complex is the main responsible of triggering the QS response by increasing the expression of a large number of genes, including those encoding the autoinducer synthase enzymes, which further increases the QSSM production. This response coordinates a number of physiological changes at a population-scale level, which allows among other issues improving bacterial competition for nutrients with other species, forming morphological-resistance structures to overcome environmental threats or triggering the expression of virulence factors (Swift and Downie, 2001; Williams, 2007). Further, it has been shown that several QSSMs can be sensed by other species promoting responses mediated by inter-species communication processes (Williams et al., 2007; Shimada et al., 2013; Lee and Zhang, 2015), including eukaryotic cells (interkingdom signaling; Martinez, 2014). As above stated these QSSMs have to cross the cells membranes for their extra-cellular accumulation, and it has been reported that some MDR efflux pumps might be involved in their transport outside the cell. In this section, we will discuss the role of MDR efflux pumps in the modulation of intercellular signaling and how the acquisition of antibiotic resistance in mutants overexpressing efflux pumps

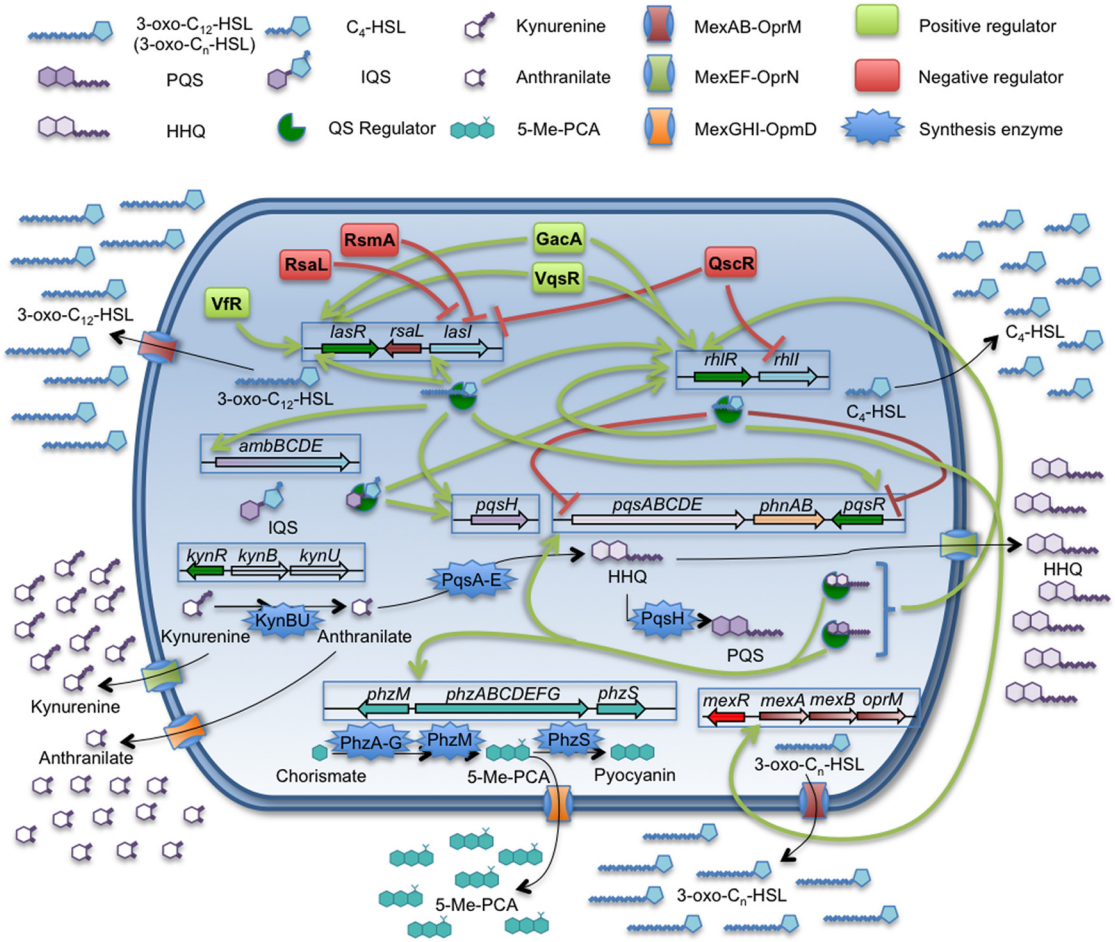

FIGURE 1 | Quorum sensing network and related efflux pumps in *P. aeruginosa*. The QS signals produced by *P. aeruginosa* are: 3-oxo-C_{12}-HSL, C_4-HSL, PQS/HHQ, and the recently discovered IQS. The figure sums up the complexity of the QS regulation network and the implications of MexAB-OprM, MexEF-OprN, and MexGHI-OpmD efflux pumps on the extrusion of QSSMs, their precursors or molecules which expression is QS-regulated. Despite of C_4-HSL is able to cross the cell envelopes by free diffusion, the expression of *mexAB-oprM* is triggered in presence of this autoinducer. Further, this efflux pump is able to extrude 3-oxo-C_{12}-HSL and others related 3-oxo-C_n-HSL. The MexEF-OprN system is able to extrude HHQ and kynurenine, a precursor of 4-alkyl-quinolones, having an impact on the QS response. In the case of MexGHI-OpmD, its role has been recently linked to the extrusion of 5-Me-PCA, a precursor of the phenazine pyocyanin, whose production is induced upon the QS response. However, it has been proposed that this system is able to efflux anthranilic acid, another AQs intermediate, which is toxic for the cell at high concentrations. *P. aeruginosa* is a good example for the potential role of efflux systems in modulating the cell-to-cell communication networks.

may challenge bacterial virulence through alterations in the diffusion of QS signals.

Pseudomonas aeruginosa is one of the most important opportunistic pathogens causing infections at hospitals as well as in cystic fibrosis patients (Buhl et al., 2015; Kaye and Pogue, 2015; Oliver et al., 2015; Talwalkar and Murray, 2016). This pathogen is able to produce different virulence factors, many of them being regulated by a hierarchically organized QS signaling system (**Figure 1**), which consists in three different and interconnected regulatory networks each one respectively governed by the transcriptional regulators LasR, RhlR, and PqsR (Lee and Zhang, 2015). These three QS regulators recognize respectively one of different QSSMs produced by *P. aeruginosa*: *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), *N*-butanoyl-L-homoserine lactone (C4-HSL), and *Pseudomonas* quinolone signal (PQS). The presence of an additional regulatory QS network has been recently described (Lee et al., 2013; Lee and Zhang, 2015). Despite being tightly regulated by the *las* system in standard conditions of growth, this new system is able to trigger the PQS and C4-HSL production in absence of the *las* system or under phosphate stress conditions. However, the mechanisms responsible of the regulation mediated by this QS system are not fully understood and consequently will not be discussed along the present review.

Some works have shown that the expression of different efflux pumps encoded in the *P. aeruginosa* chromosome may have an impact in the QS-regulation networks of this microorganism. For instance, the RND efflux pump MexAB-OprM is highly integrated within the *las* and *rhl* QS regulons, since it has

been shown that its expression can be induced by C4-HSL (Maseda et al., 2004; Sawada et al., 2004). In addition, this efflux pump is able to extrude the 3-oxo-C12-HSL QS signal (Evans et al., 1998; Pearson et al., 1999; Minagawa et al., 2012). In such a way, the constitutive high-level expression of MexAB-OprM entails the extrusion of 3-oxo-C12-HSL, increasing the concentration of this QS signal around the cell, but reducing its intracellular accumulation. Indeed, antibiotic resistant mutants overexpressing MexAB-OprM present defects in the production of several virulence factors and are impaired in their QS response (Evans et al., 1998).

It is important to highlight that the 3-oxo-C12-HSL autoinducer is not just an intra-specific signal compound but may also entail inter-kingdom signaling. For instance, it has been reported that this QS signal may inhibit the filamentous differentiation, which is linked to a virulent state of *Candida albicans*, a fungal pathogen commonly found in patients with *P. aeruginosa* infections (Hogan et al., 2004). Conversely, it has been also shown that the *C. albicans* QS compound farnesol in turn can inhibit the production of QSSMs and the production of virulence factors by *P. aeruginosa* (Méar et al., 2013), evidencing the relevance of these cell-to-cell communication "weapons" in the establishment of a competitive interaction between these two opportunistic pathogens. In addition to its role in inter-microbial interactions, a role as activator of the human immune system has been attributed to 3-oxo-C12-HSL. This autoinducer signal can act as a chemoattractant for polymorphonuclear neutrophils and is able to induce the expression of adhesion proteins and immunoglobulin receptors implicated in the recognition and localization of microbial infections (Smith et al., 2001; Zimmermann et al., 2006; Wagner et al., 2007). On the other hand, it has been shown that 3-oxo-C12-HSL can induce the apoptosis of neutrophils and macrophages (Tateda et al., 2003). The fact that 3-oxo-C12-HSL has been detected directly in the sputum of cystic fibrosis patients with *P. aeruginosa* infections indicates that this QS signal might be involved in *in vivo* interkingdom signaling (Erickson et al., 2002; Middleton et al., 2002).

Pseudomonas aeruginosa MDR strains overexpressing MexAB-OprM produce lower amounts of virulence factors as well as of 3-oxo-C12-HSL (Evans et al., 1998). In addition, *mexAB-OprM* defective mutants are avirulent in a mouse model of infection as well as in MDCK cells (Hirakata et al., 2002). These results support the notion that this efflux system is directly involved in the appropriate and coordinate *las* response needed for a successful host infection. Further, it has been proposed that this system is used by *P. aeruginosa* to optimize indirectly the specific binding of the LasR regulator to 3-oxo-C12-HSL by extruding other 3-oxo-Cn-HSL, which can affect the *las* network activation because they are able of competing with 3-oxo-C12-HSL for the same binding site in LasR (Minagawa et al., 2012). All these intra- and interspecific responses mediated by 3-oxo-C12-HSL suggest that MexAB-OprM is an important element modulating cell-to-cell communication and host–pathogen interactions. As a consequence, the acquisition of antimicrobial resistance through the overexpression of this system could have an impact, at several levels, on the virulence of *P. aeruginosa*.

Besides MexAB-OprM, other *P. aeruginosa* efflux pumps might be involved in the regulation of the expression of QS-dependent virulence factors. One of them is MexEF-OprN, an efflux pump able to extrude both the QS signal HHQ (Lamarche and Deziel, 2011) and its precursor, kynurenine (Olivares et al., 2012), making it an important element in the QS response (Köhler et al., 2001). As we have mentioned above, the virulence and pathogenicity of *P. aeruginosa* is partially controlled by the PQS communication system. Therefore, it is not surprising that, as it happens in the case of MexAB-OprM, mutants overexpressing MexEF-OprN are affected in the production of QS-regulated virulence factors and, in consequence, they are impaired in the host infection process (Olivares et al., 2012). It is worth mentioning that the extrusion of kynurenine by this system could have an additional role in *P. aeruginosa*–host interaction. It has been shown that the expression of the MexEF-OprN system can be induced upon contact with human airway epithelial cells (Frisk et al., 2004). On the other hand, it has been suggested that *P. aeruginosa* production of kynurenine may have a role in the bacterial resistance to the toxic reactive oxygen species (ROS) produced by neutrophils, a key cell component in the innate immune system and in the inflammatory response in lung infections (Genestet et al., 2014). Based on this situation, it is possible that the increased expression of MexEF-OprN upon contact with the lung epithelial cells may allow *P. aeruginosa* to secrete high levels of kynurenine, thus promoting resistance against the neutrophil ROS production. This possibility, which has not been explored yet, would provide MexEF-OprN a new role in the infected lungs beyond the modulation of the QS response.

Another *P. aeruginosa* efflux pump with potential relevance in the virulence of this pathogen is MexGHI-OprD (Aendekerk et al., 2005; Dietrich et al., 2006). It has been shown that this efflux pump is able of extruding 5-methylphenazine-1-carboxylate (5-Me-PCA), a precursor of the phenazine pyocyanin, and anthranilate, the immediate precursor of PQS (Aendekerk et al., 2005). Further, the *mexGHI-oprD* expression could be induced by 5-Me-PCA and, in consequence, is under the transcriptional control of the QS response. It is worth mentioning that the phenazine extruded by this efflux pump is required for biofilm development by *P. aeruginosa* (Sakhtah et al., 2016). If we take into consideration that biofilms are the regular way of growing of *P. aeruginosa* in the lungs of chronically infected patients (Martinez-Solano et al., 2008; Wagner and Iglewski, 2008), this indicates that MexGHI might have a relevant role in the adaptation of *P. aeruginosa* for colonizing that habitat.

Another relevant pathogen whose virulence has been associated to the extrusion of QS signals by efflux pumps is *Burkholderia pseudomallei*, the causal agent of melioidosis. Different works have suggested that the virulence of this pathogen is modulated by the stationary phase sigma factor RpoS in addition to the regulatory activity of a QS system based on both acyl-homoserine lactones (AHLs) and 2-alkyl-4-quinolones (Ulrich et al., 2004; Wongtrakoongate et al., 2012; Butt et al., 2016). *B. pseudomallei* produces up to six different homoserine lactones: *N*-octanoyl-homoserine lactone (C8-HSL), *N*-decanoyl-homoserine lactone

(C10-HSL), *N*-(3-hydroxy)-octanoyl-homoserine lactone (3-OH-C8-HSL), *N*-(3-hydroxy)-decanoyl-homo-serine lactone (3-OH-C10-HSL), *N*-(3-oxo)-decanoyl-homoserine lactone (3-oxo-C10-HSL), and *N*-(3-oxo)-tetradecanoyl-homoserine lactone (3-oxo-C14-HSL). It has been reported that in the clinical strain KHW, the BpeAB-OprB efflux pump is strictly needed for the synthesis and full extrusion of these six AHLs and it has been suggested that another efflux pump, AmrAB-OprA could also be involved in the extrusion of 3-oxo-C10-HSL (Chan et al., 2007). In agreement with a potential role of these efflux pumps in QS communication, it has been shown that *bpeAB-oprB* expression is induced in presence of exogenous C8-HSL and C10-HSL (Chan and Chua, 2005). Moreover, a *bpeAB-oprB* defective mutant is affected in the expression of QS-dependent virulence factors as well as in biofilm formation. In addition, the mutant presents impaired invasiveness and cytotoxicity in both human macrophages and lung epithelial cells. Even though the role of BpeAB-OprB in virulence might be strain-dependent (Mima and Schweizer, 2010), all of the aforementioned results support the hypothesis that some efflux systems could be involved in the modulation of *B. pseudomallei* virulence and in the host–pathogen interactions through the extrusion of QS communication signals or by responding to their presence.

Other signaling networks involved in intra- and inter-specific communication are based in the use of indole and its derived compounds as signal molecules (Bommarius et al., 2013; Shimada et al., 2013; Lee J.H. et al., 2015). Indole is synthesized by plants and by many different bacteria and has different roles depending on the target species. Even though it was proposed that the efflux pump AcrEF could be implicated in indole export in *Escherichia coli* (Kawamura-Sato et al., 1999), other works have shown that indole is able to diffuse easily across the bacterial envelope (Gaede et al., 2005; Pinero-Fernandez et al., 2011) so that a clear role of efflux pumps in indole trafficking remains controversial. However, a crosstalk between virulence and efflux pumps-linked antibiotic resistance mediated through indole signaling is possible. Indeed, indole may affect the expression of efflux pumps, the resistance to antibiotics and the behavior of *Salmonella enterica* serovar Typhimurium when growing inside the host (Nikaido et al., 2012). This bacterial pathogen does not produce indole. However, it usually lives together with indole-producing species in the host and is able to respond to this signal (Nikaido et al., 2011). It is proposed that indole blocks the activity of the RamR transcriptional regulator through the interaction with its C-terminal domain, leading to the overexpression of RamA activator (Nikaido et al., 2011, 2012). This regulatory protein is able to induce the expression of *acrAB*, *acrEF*, and *tolC*, in addition of repressing the expression of some virulence determinants (Bailey et al., 2010; Nikaido et al., 2012). This entails the development of a low-virulence phenotype, which presents, however, increased resistance to antibiotics as well as to host-produced toxic compounds as bile salts and fatty acids, a phenotype closer to a commensal behavior than to an infective one. This is another example of the relationship between the communication signals, with inter-specific function in this case and the expression of efflux pumps, a situation with clear implications in the virulence potential of bacterial pathogens. Indeed, the role of AcrAB-TolC in the virulence of *Salmonella* goes beyond the acquired phenotype upon induction by indole, since, as described below, it has been shown that *acrAB-tolC* deficient mutants have reduced invasiveness in animal models (Buckley et al., 2006; Webber et al., 2009).

MULTIDRUG EFFLUX PUMPS AND THEIR ROLE IN THE VIRULENCE OF HUMAN PATHOGENS

During the course of an infection, a bacterial pathogen has to be capable of surviving from the anti-infective defense mechanisms of the host. These mechanisms include, among others, the production of a diverse set of antimicrobial compounds as fatty acids, peptides or even detergents as bile salts, which function is in food uptake, but also present antimicrobial activity (Fernando and Kumar, 2013). In addition of extruding antibiotics regularly used for treating bacterial infections, different MDR efflux pumps have the ability to extrude a wide variety of compounds, including those antimicrobials produced by the host as well as QS signals involved in the regulation of the expression of virulence determinants (see above). Consequently, these efflux pumps are relevant payers on both antibiotic resistance and virulence of bacterial populations (Piddock, 2006b; Martinez et al., 2009; Alvarez-Ortega et al., 2013).

In the case of enteric bacteria, it has been shown that the efflux of several host-derived antimicrobial compounds, such as bile salts, allows the colonization and promotes the bacterial adaptation to the animal intestinal tract. The best-studied system able to confer resistance to bile salts is the *E. coli* RND efflux pump AcrAB-TolC, which is also a major contributor to intrinsic resistance to antibiotics in this organism (Thanassi et al., 1997). Similar roles have been reported for different AcrAB homologs from other Enterobacteriaceae species, such as *S. enterica* serovar Typhimurium. In this case, it has been described that mutants lacking *acrB* and *tolC* are less proficient for adhering, invading and surviving in mouse monocyte macrophages. The same study reported that an *acrB* mutant was able to colonize chicks. However, it was unable to survive gastrointestinally, which suggests that AcrB is not particularly relevant for the early steps of the gut colonization but it is needed for gastrointestinal persistence. On the other hand, the *tolC* mutant was able to colonize and persist in the chicken intestinal tract, but with a much lower efficiency than the wild-type strain, which could be due to the bile hypersensitivity displayed by this mutant (Buckley et al., 2006). A more recent study, using total genome transcriptional analysis, showed that the inactivation of *acrA*, *acrB*, and *tolC* rendered changes in the level of expression of several genes involved in bacterial pathogenicity, further supporting a crosstalk between resistance and virulence (Webber et al., 2009). For instance, the disruption of *acrB* or *tolC* led to a general repression of the SPI-1 pathogenicity island, which promotes the invasion of non-phagocytic intestinal epithelial cells, as well as bacterial survival and persistence within the host, while inactivation of *acrA* is associated with a repression of SPI-2, which promotes the survival and multiplication in

phagocytic cells (Dieye et al., 2009). Therefore, inactivation of *acrB* led to the inability to grow anaerobically, which would negatively impact in the capacity to survive in the host gut; as well as a reduced bacterial motility (Webber et al., 2009), which is also an important factor in the *S. enterica* serovar Typhimurium pathogenicity (Khoramian-Falsafi et al., 1990). As above stated, RND efflux systems are tripartite protein complexes formed by an inner membrane protein (the pump itself), an outer membrane protein and a linker, membrane fusion protein (Yamaguchi et al., 2015; Daury et al., 2016). The inner membrane and the membrane fusion proteins are always encoded in the same operon and are supposed to be specifically associated in each efflux pump, while the outer membrane protein can frequently form part of different efflux pumps. Consequently, while a differential response to the inactivation of the outer membrane protein of the complex (TolC in this case) as compared with the other members of the complex is conceivable, the observed differences between the *acrA* and *acrB* are more difficult to explain and suggests that a certain degree of trans-complementation between the components of different efflux pumps may happen. Although some information on this possibility has been published (Smith and Blair, 2014), this is a topic that remains to be studied in detail, although it merits to be addresses if we wish to understand in full the role that efflux pumps may play in the bacterial physiology besides antibiotic resistance.

An homologous of AcrAB is also present in the respiratory tract pathogen *Moraxella catarrhalis*. In addition of contributing to antibiotic resistance, this efflux pump is also involved in the efficient invasion of nasopharyngeal epithelial cells, since mutants lacking *acrA*, *acrB*, or *tolC* present decreased invasion levels as compared to the wild-type strain (Spaniol et al., 2015). This study also showed that exposure to cold shock (26°C) led to an increase in the expression of the efflux pump genes (Spaniol et al., 2015). Since temperature is an important factor for the adaptation and survival in the respiratory tract, as well as for the colonization properties and the virulence of *M. catarrhalis* (Heiniger et al., 2005; Spaniol et al., 2013), these results indicate that the levels of expression of this efflux pump are controlled by cues with relevance for the infectious success of *M. catarrhalis*. This further supports that efflux pumps may form part of global regulatory networks that include resistance and virulence determinants among other elements (Randall and Woodward, 2002; Luong et al., 2003; Nikaido et al., 2008; De Majumdar et al., 2013).

The ability to persist and to replicate in bile-rich environments is also critical for the pathogenesis of the food-borne pathogen *Listeria monocytogenes*. Among the elements that contribute to its survival in bile salts are the MDR efflux pumps MdrM and MdrT, belonging to the MFS family (Quillin et al., 2011). Expression of both MDR efflux pumps is strongly induced by cholic acid, a bile component, but only the MdrT efflux pump is able to extrude this compound, which is toxic for mutants lacking this efflux pump. Besides, MdrT is an important virulence factor involved in the colonization of the gallbladder *in vivo*, since mutants lacking *mdrT* are 100-fold attenuated. This study also suggests that MdrM has a synergistic role with MdrT in *L. monocytogenes* liver colonization, although MdrM substrates have not been identified yet (Quillin et al., 2011).

RND efflux systems also play a role in the pathogenesis of *Vibrio cholerae*. It has been reported that VexAB, VexCD, VexIJK, and VexGH contribute not only to antimicrobial resistance, but also to the colonization of the infant mouse small intestine; deletion of these systems impair the colonization of the mice intestine by *V. cholerae* (Bina et al., 2008; Taylor et al., 2012). Besides being relevant factors for colonization, these efflux pumps are required for the expression of the genes that encode two of the most important virulence factors in *V. cholerae*: the cholera toxin (CT) and the toxin-coregulated pilus (TCP). In a mutant lacking *vexB*, *vexD*, *vexH*, and *vexK*, the production of CT and TcpA (the pilin subunit of the TCP) is reduced by 45%, while a six RND-null strain showed a 70% reduction in the expression of these virulence determinants, suggesting that the remaining efflux pumps, VexF and VexM, also contribute to virulence in *V. cholerae* (Taylor et al., 2012).

Multidrug efflux systems are also relevant elements in the defense against oxidative stress produced in the host during phagocytosis. For example, the ABC family efflux pump MacAB is required for the survival of *S. enterica* serovar Typhimurium inside macrophages, where they are exposed to ROS. It has been observed that mutants lacking *macAB* showed an impaired intracellular replication in macrophages as compared with the wild-type parental strain and also failed in growing in the inflamed intestine, where neutrophils release ROS. Further, the same mutants were able to grow inside macrophages that do not produce ROS, which implies that MacAB is needed for *S. enterica* serovar Typhimurium replication inside macrophages and for the survival under oxidative stress conditions. Besides, the *macAB* deletion mutant had a defect in liver colonization in BALB/c mice after intraperitoneal infection, which indicates another function of this system in the infective program of *S. enterica* serovar Typhimurium (Bogomolnaya et al., 2013).

In addition to the role of efflux pumps in the interaction of bacterial pathogens with the compounds present in the host, they also may play a direct role in virulence. This is the case for the *Mycobacterium tuberculosis* RND proteins designated as MmpL (*M*ycobacterial *m*embrane *p*rotein *L*arge; Cole et al., 1998). The genome of *M. tuberculosis* encodes 13 of these proteins, which role seems to be transporting lipids for their incorporation on the cell envelope, providing protection against host-derived compounds and contributing to the bacterial virulence (Neyrolles and Guilhot, 2011). Domenech et al. (2005) examined the contribution of these proteins to the bacterium virulence by using a murine model of infection and mutants for each MmpL protein. Among these proteins, they found that MmpL4, MmpL7, MmpL8, and MmpL11 were required for the virulence maintenance, since there is an increase in the survival time when the host is infected with these mutants as compared with the wild-type strain. The attenuation of the *mmpL7* mutant might be due to the lack of phthiocerol dimycocerosate (PDIM), which is an abundant wax of the outer cell matrix involved in the cell permeability (Camacho et al., 2001); and the *mmpL8* mutant is deficient in SL-1 because MmpL8 is involved in the transport of SL-N, a precursor of

SL-1 (Domenech et al., 2004). It has been shown that SL-N stimulates human CD1b-restricted T cells (Gilleron et al., 2004), a feature that might explain the attenuation of the *mmpL8* mutant if this molecule has a similar effect on murine CD1d-restricted T cells. The mechanisms by which *mmpL4* and *mmpL11* mutants were more attenuated have not been fully elucidated; however, it has been recently described a mutation in *mmpL4a* (Tyr842His) in *Mycobacterium bolletii*, which is responsible for the smooth-to-rough morphotype change, since MmpL4 is involved in the transport of glycopeptidolipid. This variant also contributes to the bacterial virulence in a zebrafish model (Bernut et al., 2016). The Tyr842 residue is conserved in all other mycobacterial MmpL4 orthologs and in all 13 MmpL RND proteins in *M. tuberculosis*, which indicates the functional relevance of this residue (Szekely and Cole, 2016).

Biofilms are complex microbial associations attached to a variety of surfaces. Bacteria that grow forming biofilms are more resistant to antibiotics than planktonic cells, being also important elements in the bacterial virulence and pathogenesis. The link between antimicrobial tolerance on biofilms and efflux pumps has been reported in several microorganisms (Soto, 2013). For instance, in the opportunistic pathogen *P. aeruginosa*, the MerR-like regulator BrlR plays a role in the high-level tolerance to antimicrobials in biofilms because it is able to activate under these growing conditions the expression of the multidrug efflux pumps MexAB-OprM and MexEF-OprN (Liao et al., 2013). A novel efflux pump in *P. aeruginosa* involved in biofilm tolerance, named as PA1874-1877, has been identified. The expression of this efflux pump was 10-fold increased in biofilms when compared with planktonic cells. Besides, deletion of the genes encoding this efflux pump resulted in an increased susceptibility to ciprofloxacin, gentamicin, and tobramycin (Zhang and Mah, 2008). Efflux pump expression can also impact the flagellar motility, which plays a relevant role in biofilm formation (Houry et al., 2012) and enhances pathogenicity by improving bacterial motility (Duan et al., 2013). In *Stenotrophomonas maltophilia*, another opportunistic pathogen, it has been observed that deletion of the RND efflux pump SmeYZ resulted in a reduced ability to form biofilm and the abolition of flagella formation. Besides, this deletion mutant was more susceptible to redox compounds, human serum and neutrophils, which indicates that this efflux pump is also involved in the protection against ROS (Lin et al., 2015).

Expression of efflux pumps not always enhance virulence; constitutive overexpression of these systems in antibiotic resistant mutants, can compromise the bacterial fitness and the virulence as well (Sanchez et al., 2002), indicating that the expression of these elements is finely regulated and deviations on this regulation, altering their expression below or above the physiological levels, may impair bacterial physiology and virulence. This is the case of the overexpression of the MDR efflux pumps MexCD-OprJ and MexEF-OprN in *P. aeruginosa*, which negatively affect the expression of the type III secretion system (T3SS; Linares et al., 2005). The T3SS is an important virulence mechanism, since bacteria are able to inject effector proteins manipulating the host cell function (Coburn et al., 2007). The effect of over-expression of efflux pumps on T3S was due to the lack of expression of *exsA* gene, which is the transcriptional activator of the T3SS in *P. aeruginosa* (Linares et al., 2005). More recently, it was found that MexT, the positive regulator of MexEF, was able to repress the expression of the T3SS through the regulators MexS and PtrC (Jin et al., 2011), in such a way linking resistance and virulence within a single regulon.

Altogether, these works show that, in addition of being involved in antibiotic resistance, efflux pumps can participate in bacterial virulence as well. In some cases, a direct effect can be foreseen; this is the situation of efflux pumps able to extrude host-produced antimicrobial compounds. However, in other occasions the reasons behind the effect of efflux pumps on virulence are not so straightforward. In some cases, the effect on virulence is derived from the integration of the efflux pumps in a regulon that also includes virulence determinants. This could be the case of MexEF, a part of the MexT regulon that also includes the *P. aeruginosa* T3SS. Mutations in this global regulator will simultaneously alter antibiotic resistance and virulence, although the efflux pump itself is not directly involved in T3S. Other situation, also described for MexEF, is the capability of some efflux pumps for extruding intercellular signal molecules (see above) or their precursors (Olivares et al., 2012). If this efflux pump is abnormally expressed, the levels of expression of the genes belonging to the regulatory network (frequently including virulence genes) triggered by such signals will be also altered.

THE FUNCTIONAL ROLE OF MULTIDRUG EFFLUX PUMPS IN PLANT–BACTERIA INTERACTIONS

Multidrug efflux pumps, in addition of being relevant antibiotic resistance determinants, are relevant key players for the behavior of microorganisms in their natural (non-clinical) habitats. Indeed, whereas most studies in human pathogens have concentrated in the role on antibiotic resistance of these elements, the analyses of efflux pumps from plants pathogens or epiphytes, has mainly focused on their role in plant–bacteria or bacteria–bacteria interactions. The rhizosphere is a natural ecosystem that includes a complex microbiome formed by microorganisms that live in contact with plants' roots. Roots' and other plants' exudates contain a large array of natural products, such as flavonoids, which confer them protection against microbial attack. However, different microorganisms have developed mechanisms to deal with the activity of those compounds. One of them is the flavonoid-responsive RND family of efflux pumps, which includes several members as MexAB-OprM from *Pseudomonas syringae*, AcrAB from *Erwinia amylovora*, AcrD from *Erwinia chrysanthemi*, IfeAB from *Agrobacterium tumefaciens*, XagID2689 from *Xanthomonas axonopodis*, SmeDEF from *S. maltophilia*, EmrAB from *Sinorhizobium meliloti* and BjG30 from *Bradyrhizobium japonicum* (Palumbo et al., 1998; Burse et al., 2004b; Vargas et al., 2011; Takeshima et al., 2013; García-León et al., 2014; Pletzer and Weingart, 2014; Rossbach et al., 2014; Chatnaparat et al., 2016). Some of these efflux pumps are implicated in plant colonization, whereas

some others are involved in bacteria/plant symbiosis processes. As stated before MexAB-OprM is described as a significant determinant of multidrug resistance in *P. aeruginosa* (Poole, 2001) having a basal expression level enough to contribute to the intrinsic antimicrobial resistance of these bacteria (Li et al., 1995) and it also has a role in virulence. Indeed, as above described, mutant strains overexpressing this efflux pump are less virulent because of the extrusion of the QS homoserine lactone 3-oxo-C_{12}-HSL (Minagawa et al., 2012). In addition of extruding antibiotics or QS signaling molecules, MexAB-OprM is able to pump out plant antimicrobial compounds from leaves of *Melaleuca alternifolia*, supporting that, in addition of its role in clinical settings, this efflux pump (and several others, see below) may allow bacterial survival in a vegetal environment (Papadopoulos et al., 2008). Indeed, this efflux pump is required for the efficient colonization of tomato plants by *P. syringae*, since the inactivation MexAB-OprM lead to a defective colonization capacity of the plant by this bacterial species (Vargas et al., 2011). In line with the role of efflux pumps in a general bacterial response to the plant antimicrobial defense, it has been shown that flavonoids are inducers and substrates of this transporter (Vargas et al., 2011). In addition, the same flavonoids are able to inhibit the GacS/GacA two component system (TCS) of *P. syringae*, which is implicated, among other regulatory elements, in the activation of the motility and the T3SS in this species (Chatterjee et al., 2003). In fact, the absence of MexAB-OprM and the accumulation of flavonoids inside bacteria lead to a reduction of swarming and swimming motility and a significant impairment in the production of flagella and T3S (Vargas et al., 2013). Therefore, when the amount of plant flavonoids is enough to reduce bacterial virulence by inhibiting GacS/GacA, *P. syringae* may extrude such flavonoids using the MexAB efflux pump (which expression is now induced), shrewdly regulating the intracellular level of flavonoids and consequently, ensuring the viability of the bacteria by the de-repression of this TCS. This is an elegant example of the adaptive co-evolution of plant resistance and pathogen virulence in which the role of this efflux pump goes beyond being a mere detoxification system (**Figure 2**).

Another plant-pathogenic bacteria is *E. amylovora*, an Enterobacteria that causes fire blight on apple and pear trees. The flavonoid inducible AcrAB-TolC efflux pump from *E. amylovora* confers resistance, in addition to antibiotics, to plant compounds as phytoalexins and flavonoids (phloretin, naringenin, and quercetin) leading to the successful colonization of plants (Burse et al., 2004b; Maggiorani Valecillos et al., 2006; Al-Karablieh et al., 2009). Moreover, an AcrAB deletion mutant has a strong reduction of *E. amylovora* virulence in apple plants (Burse et al., 2004b). A close homolog of AcrB is AcrD, an efflux pump that is also induced by plant flavonoids, such as luteolin, but whose deletion mutant exhibits full virulence on apple and pear fruits (Pletzer and Weingart, 2014). *E. chrysanthemi* constitutes another example in which efflux pumps are required to infect plants. A *tolC* mutant of *E. chrysanthemi* is unable to extrude the plant antimicrobial compound berberine, being then unable to cause plant tissue maceration. Moreover, TolC plays a role also modulating the fitness of the bacteria growing in the microbial community (Barabote et al., 2003), indicating that efflux pumps might also be involved in inter-microbial interactions (see below). In addition of plant-derived antimicrobials, the AcrAB efflux pump of *E. chrysanthemi* is inducible by salicylic acid (SA), an important plant hormone implicated in local and systemic plant resistance (Ravirala et al., 2007). SA and others plant phenolic acids are able of reducing the expression of the T3SS, by inhibiting the GacS/A pathway, in *P. syringae* (Lee J.S. et al., 2015), suggesting that a role of efflux pumps in non-clinical ecosystems might be to adjust the intracellular level of plant signals, as a mechanism to deal with plant defense systems. Other efflux pump that has a role in plant colonization is IfeAB from *A. tumefaciens*, which extrudes coumestrol bestowing measurable ecological benefits to this bacterium in flavonoids rich environments. *X. axonopodis* pv. *glycines* is another plant pathogen, causative of bacterial pustule of *Glycine max*, one of the most important diseases in soybean. It contains another flavonoid inducible RND efflux pump, called XagID2689 (Chatnaparat et al., 2016), which in addition of being induced by flavonoids contributes as well to flavonoids' resistance; its deletion strongly reduces bacterial virulence in soybean. Moreover, this deletion mutant shows a higher susceptibility than the wild-type parental counterpart to the isoflavonoids phloretin, naringenin and berberine, as well as to the antibiotics acriflavine and tetracycline, suggesting a role in the intracellular reduction of the levels of several compounds produced by soybean and involved in its antimicrobial response program. In *S. maltophilia*, the SmeDEF efflux pump, which is the most important quinolone resistance determinant of this microorganism (Alonso and Martinez, 2000, 2001; Zhang et al., 2001; Garcia-Leon et al., 2014), is induced by flavonoids, that are able to bind to its SmeT repressor (García-León et al., 2014); in addition, a mutant lacking *smeE* is unable to colonize the roots of *Arabidopsis* plants (García-León et al., 2014), further supporting that MDR efflux pumps, with a relevant role for antibiotic resistance at clinical settings, might have been selected in nature for different ecological purposes.

In addition of easening the bacterial plants' colonization and infection, efflux pumps have also a role in the interactions between plants and their symbiotic bacteria. For example, EmrAB from *S. meliloti* is an inducible flavonoid efflux pump with a role in symbiosis with *Medicago sativa*; the symbiotic process is impaired when the regulator of this efflux system, the TetR repressor EmrR, is deleted (Rossbach et al., 2014; Santos et al., 2014). Other examples of efflux pumps with a role in symbiotic nitrogen-fixation activity processes in *G. max* are BdeAB and BjG30 from *B. japonicum* (Lindemann et al., 2010; Takeshima et al., 2013). BdeAB deficient mutants, in addition of presenting symbiotic defects, are more susceptible to aminoglycosides, highlighting the multifunctional role of efflux pumps (Lindemann et al., 2010).

Efflux pumps have also an important role in the inter-microbial interactions in the host plant and in its rhizosphere, where each bacteria have to compete for space and nutrients to survive. As with antibiotic resistance mechanisms, that can be considered as a colonization factor in the treated

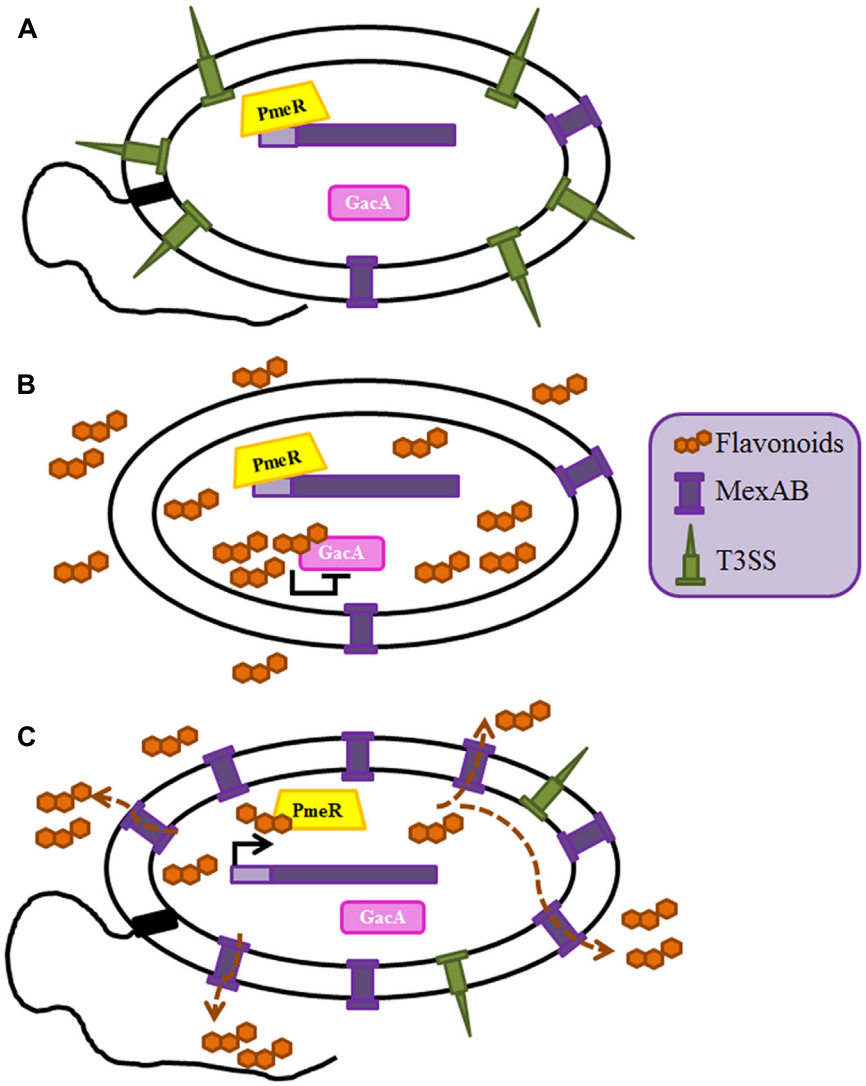

FIGURE 2 | Role of the MexAB-OprM efflux pump in the adaptive co-evolution of plant defense and *P. syringae* virulence. The intracellular amount of the plant flavonoids is regulated by MexAB-OprM efflux pump. **(A)** Expression of *mexAB-oprM* is controlled by the transcriptional repressor PmeR; in the absence of flavonoids the expression of this efflux pump is repressed and expression of virulence determinants as the T3SS or bacterial motility is activated through the GacS/A TCS (Vargas et al., 2011). **(B)** The presence of flavonoids inhibits GacS/A, hence leading a transient low virulence phenotype, in which the expression of the T3SS and the motility are low (Vargas et al., 2013). **(C)** The accumulation of flavonoids inside the cell leads to over-expression of *mexAB-oprM* through the release of its transcriptional regulator PmeR from its operator. Since flavonoids are substrates of MexAB-OprM, under these conditions they are efficiently extruded and their intracellular concentration falls down below the threshold required for inactivating GacS/GacA. As a consequence of this situation, the virulent state of *P. syringae* is restored.

patient, where antibiotics are present (Martinez and Baquero, 2002), resistance to antimicrobials produced by epiphytes may be considered as a colonization tool of phytopathogenic bacteria and *vice versa*. If the epiphytes can inhibit the phytopathogens, the plant will be protected from infection, whereas if pathogens can inhibit epiphytes, this situation will provide a colonization advantage and hence better possibilities for infecting the plant. Resistance to compounds produced by bacterial competitors will then increase the chances for colonization of the epiphyte or the pathogen, with important consequence in terms of crops protection. This is the case of the efflux pump NorM from *E. amylovora* that, in contrast to the previously mentioned efflux pump AcrAB from *E. amylovora* (Burse et al., 2004b), is not able to extrude the high amount of isoflavonoids that produce the members of the Rosaceae family (Burse et al., 2004a). Moreover, a *norM*-deficient mutant causes comparable symptoms as the wild-type parental counterpart in plant tissues, indicating that this efflux pump does not contribute to virulence of *E. amylovora* against apple plants. Nevertheless, while the NorM efflux pump does not directly contribute to virulence *E. amylovora*, it has an important role extruding toxic molecules produced by *Pantoea*

agglomerans (Pusey et al., 2011), an epiphytic bacteria that is an excellent colonizer of stigmas of apple and pear and it may effectively inhibit the multiplication of *E. amylovora* when both microorganisms co-colonize rosaceous (Burse et al., 2004a; Piddock, 2006b), hence constituting a potential biocontrol agent for fire blight (Kim et al., 2012). It is worth mentioning that NorM is involved in the capability *E. amylovora* to reach high-density populations at low temperatures. Indeed, at 18°C the growth of an *E. amylovora norM* mutant is significantly impaired as compared with the wild-type strain. In addition, the level of expression of *norM* is twofold greater at 18°C gene than at 28°C (Burse et al., 2004a). Altogether these results suggest that the intrinsic resistance of *E. amylovora* to *P. agglomerans* competition at 18°C may be due to the increased expression level of *norM*, which might allow the colonization by *E. amylovora* of the stigma surface of the blossom when it coexists with *P. agglomerans*. These evidences lead to hypothesize that the utilization of *P. agglomerans* as a biocontrol mechanism for fire blight would be compromised at low temperatures or by mutations causing of overexpression of *norM* in *E. amylovora*.

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PSM-Mec—A Virulence Determinant that Connects Transcriptional Regulation, Virulence, and Antibiotic Resistance in Staphylococci

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PSM-mec is a secreted virulence factor that belongs to the phenol-soluble modulin (PSM) family of amphipathic, alpha-helical peptide toxins produced by *Staphylococcus* species. All known PSMs are core genome-encoded with the exception of PSM-mec, whose gene is found in specific sub-types of SCC*mec* methicillin resistance mobile genetic elements present in methicillin-resistant *Staphylococcus aureus* and coagulase-negative staphylococci. In addition to the cytolytic translational product, PSM-mec, the *psm-mec* locus encodes a regulatory RNA. In *S. aureus*, the *psm-mec* locus influences cytolytic capacity, methicillin resistance, biofilm formation, cell spreading, and the expression of other virulence factors, such as other PSMs, which results in a significant impact on immune evasion and disease. However, these effects are highly strain-dependent, which is possibly due to differences in PSM-mec peptide vs. *psm-mec* RNA-controlled effects. Here, we summarize the functional properties of PSM-mec and the *psm-mec* RNA molecule and their roles in staphylococcal pathogenesis and physiology.

Keywords: *Staphylococcus aureus*, *Staphylococcus epidermidis*, phenol-soluble modulin, PSM-mec, regulatory RNA, SCC*mec*, virulence

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INTRODUCTION

Staphylococcus aureus is a serious human pathogen responsible for a multitude of human diseases, which range from acute skin and soft tissue infections to more severe illnesses such as catheter-associated bacteremia, necrotizing pneumonia, and osteomyelitis. Since the emergence of methicillin-resistant *S. aureus* (MRSA) in the early 1960s, *S. aureus* has continued to cause significant morbidity, mortality, and a considerable financial burden for public health systems. For *S. aureus* and its closely related cousin, *Staphylococcus epidermidis*, the acquisition of antibiotic resistance, coupled with the ability to attach to surfaces and form sticky, multicellular agglomerations (biofilms; Costerton, 1999) on indwelling catheters, are two defining hallmarks that account for the ongoing difficulty in treating immuno-compromised individuals (Otto, 2008) with infections caused by Hospital-Associated MRSA [HA-MRSA] (Otto, 2012) and *S. epidermidis* (Otto, 2009).

Genetic mutations and/or the acquisition of mobile genetic elements (MGEs) that harbor drug resistance genes are two common mechanisms that are responsible for antibiotic resistance in microorganisms. Arguably the most important staphylococcal MGE is *Staphylococcus* cassette

chromosome *mec* (SCC*mec*), which codes for methicillin resistance. Recently, a unique locus was discovered in specific types of SSC*mec* elements in *S. aureus* and *S. epidermidis*, which comprises a small regulatory (sr)RNA and an embedded gene encoding a cytolytic peptide called PSM-mec (Queck et al., 2009). PSM-mec belongs to the phenol-soluble modulin (PSM) family of amphipathic, alpha-helical peptide toxins produced by *Staphylococcus* species (Wang et al., 2007, 2011; Diep and Otto, 2008; Otto, 2014; Cheung et al., 2014a), which collectively play an important role as virulence determinants in many facets of *S. aureus* and *S. epidermidis* pathogenesis (Wang et al., 2011; Periasamy et al., 2012; Cassat et al., 2013; Cheung et al., 2014a). Here, we will discuss the current knowledge on the PSM-mec and the *psm-mec* srRNA molecule, with a focus on their roles in *S. aureus* pathogenesis.

THE DISTRIBUTION OF THE *psm-mec* LOCUS IN SCC*mec*

SCC*mec* elements, which range in size from 21 to 67 kb, comprise as characteristic components, the *mecA* gene that codes for a penicillin-binding protein, a *ccr* gene complex for site-specific recombination, and flanking repeat sequences for integration into the genome (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements, 2009). SCC*mec* elements are classified into 11 different "types," as defined by the presence of different *ccr* and *mec* resistance genes (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements, 2009; Li et al., 2011; Shore et al., 2011) and further divided into "subtypes" based on the presence of other resistance genes and transposons in the non-essential J regions (Ito et al., 2003). The *mec* gene complex has four core elements; IS*431*, *mecA*, *mecR1,* and *mecI*, which represent the genes coding for insertion sequence IS*431*, a modified penicillin binding protein 2a (PBP2a), the signal transducer protein, and the methicillin resistance regulatory protein (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements, 2009; Shore and Coleman, 2013; **Figure 1A**).

The *psm-mec* locus is found in the J2 region adjacent to *mecI* of the class A *mec* gene complex in several widespread HA-MRSA lineages, but most prominently in Sequence Type (ST) 5, ST36, ST45, ST225, and ST239 strains (Deurenberg and Stobberingh, 2009; Nübel et al., 2010; Castillo-Ramirez et al., 2012; Schulte et al., 2013), which all have either SCC*mec* types II or III (Chatterjee et al., 2011; Monecke et al., 2012; Josten et al., 2014). However, more detailed analysis revealed that the *psm-mec* locus is found in SCC*mec* types II, IIA, IIB, IID, III, and VIII of many *Staphylococcus* species (Monecke et al., 2012; Shore and Coleman, 2013).

Antibiotic resistance in bacteria is often associated with a reduced fitness cost to the bacterial host (Otto, 2013a). We recently reported that the expression of the PSM-mec peptide impacted oxacillin resistance in *S. aureus* (Cheung et al., 2014b), suggesting that a delicate balance may exist between virulence gene expression and methicillin resistance in MRSA strains carrying the *psm-mec* locus.

EXPRESSION AND REGULATION OF THE PSM-mec PEPTIDE

The production of the PSM-mec peptide, which can be identified in bacterial culture filtrates by high-pressure liquid chromatography/mass spectrometry (HPLC/MS) (Queck et al., 2009) or Matrix-assisted laser desorption/ionization Time of Flight (MALDI-TOF) analysis (Josten et al., 2014), is a reliable indicator of methicillin resistance. However, the restricted distribution of the *psm-mec* locus severely limits the potential use of HPLC-based detection of PSM-mec for routine clinical screening of methicillin resistance.

The expression of all PSMs including the MGE-encoded PSM-mec is positively regulated by the accessory gene regulatory (*agr*) quorum sensing system (Novick, 2003; Queck et al., 2008; Chatterjee et al., 2011). This up-regulation is dependent on the binding of activated AgrA, the response regulator of *agr*, to promoter sequences upstream of the *psm* genes (Queck et al., 2008), but this has not directly been demonstrated for the *psm-mec* promoter. Interestingly, PSM expression is also enhanced in the presence of calf serum (Oogai et al., 2011) and up-regulated within intracellular environments after the activation of the stringent response (Geiger et al., 2012). Whether this is also the case for PSM-mec is not known.

PSM-mec production was shown to be especially frequent among clinical isolates of *S. epidermidis* compared to isolates from the skin of healthy individuals (Queck et al., 2009), which is a direct consequence of the genetic connection of *psm-mec* with methicillin resistance, which occurs much more frequently in clinical isolates. However, expression levels of PSM-mec can vary strongly among isolates (Queck et al., 2009). The mechanistic reasons for varying levels of PSM-mec expression between isolates cannot be fully explained but a documented -7T > C mutation in the *psm-mec* promoter is known to diminish PSM-mec expression in a subset of HA-MRSA strains (Kaito et al., 2011, 2013; Aoyagi et al., 2014).

CHARACTERIZATION OF PSM-mec PEPTIDE

Like all members of the PSM family, PSM-mec is expressed with an N-terminal formyl-methionine and secreted without a signal peptide (Wang et al., 2007; Queck et al., 2009). Although this has not yet been shown directly for PSM-mec, it is likely that it is also exported by one or both of the two dedicated ATP-Binding Cassette (ABC) transporters that secrete PSMs (Chatterjee et al., 2013; Yoshikai et al., 2016). The 22 amino acid PSM-mec fits into the class of shorter α-type PSMs, which are anywhere between∼ 20–25 amino acids in length, as opposed to the β-type, which are approximately twice as long (43–45 amino acids) (Cheung et al., 2014a).

PSM-mec has a unique cysteine residue at position 17, while cysteine residues are not present in the sequences of any other

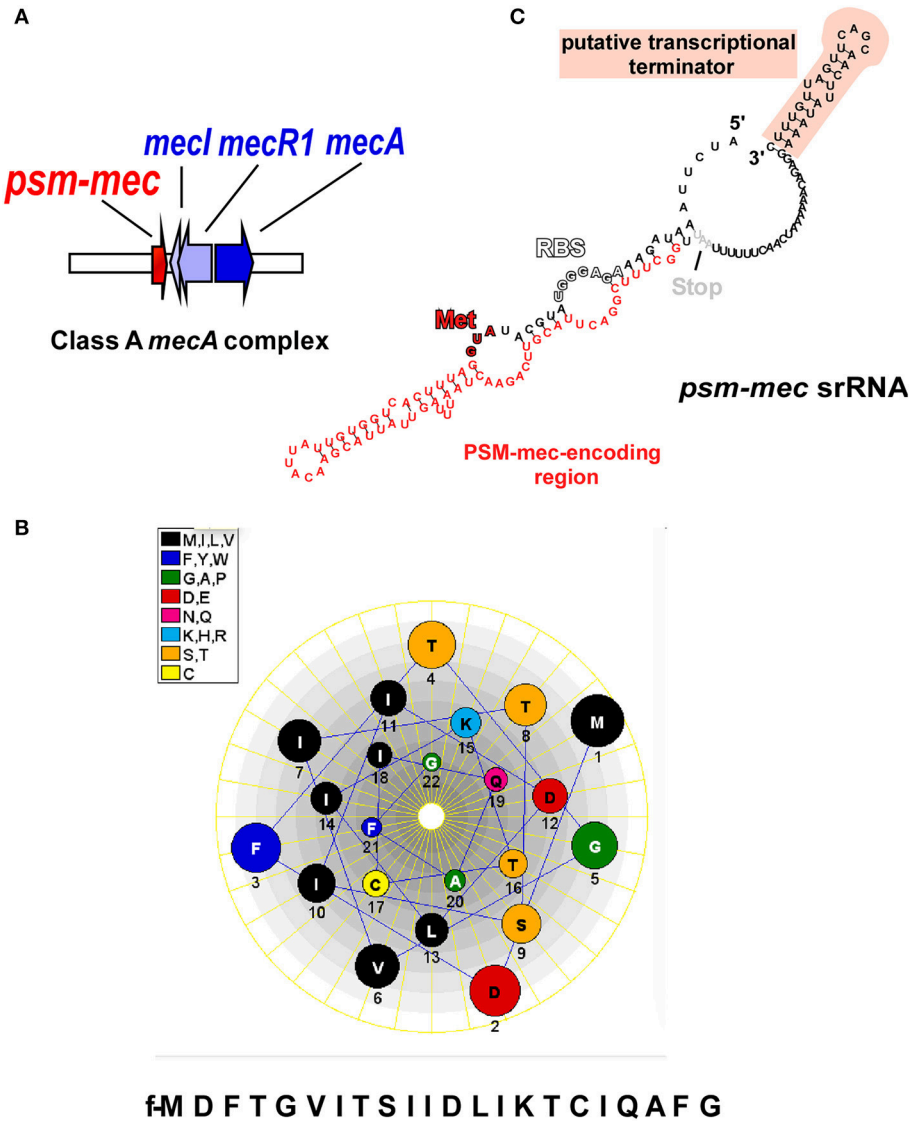

FIGURE 1 | The *psm-mec* locus and its products. (A) The *psm-mec* gene is located on the class A *mecA* complex in SCC*mec* elements of types II, III, and VIII, next to the *mecI*/*mecR1*/*mecA* genes that confer methicillin resistance and its regulation. **(B)** The PSM-mec peptide product of the *psm-mec* locus forms an α-helix with pronounced amphiphathy; helical wheel presentation. **(C)** The *psm-mec* gene is part of a regulatory RNA with pronounced predicted secondary structure. The *psm-mec* gene is entirely embedded in a highly folded, large stem loop. The *psm-mec* srRNA only slightly extends toward the 3′ end and ends with a characteristic predicted terminator stem loop structure (Note, however, that Kaito et al. reported the srRNA to be slightly longer at the 5′ end). RBS, ribosomal binding site; Start codon coding for (N-formyl) methionine.

known PSM (**Figure 1B**). The notion of a hypothetical dimeric form of PSM-mec, due to the formation of disufide bridges created from oxidized cysteine residues, was formally discounted after size exclusion chromatography experiments showed no difference in extracted ion chromatograms of PSM-mec mutants compared to that of the unaltered PSM-mec peptide (Queck et al., 2009). However, the cysteine residue was crucial for pro-inflammatory and cytolytic activity, probably because it is essential for the PSM-mec secondary structure (Queck et al., 2009).

THE PRO-INFLAMMATORY AND CYTOLYTIC PROPERTIES OF PSM-mec

At nanomolar concentrations, the PSMs are powerful pro-inflammatory agents and have an extraordinary capacity for activating and stimulating neutrophils (Wang et al., 2007), which is exerted through their interactions with human formyl peptide receptor 2 (FPR2; Kretschmer et al., 2010), a G protein-coupled receptor expressed on multiple immune cell types (Migeotte et al., 2006). Although differences in activity are

observed between PSM members, PSM-mec readily up-regulates both CD11b and gp91phox expression, induces calcium flux, chemotaxis, and IL-8 release in neutrophils (Queck et al., 2009).

On the other hand, several PSM members of the α-type possess potent cytolytic activities, and an ever-increasing number of immune and non-immune cells are being discovered that are subject to PSM-mediated cytolysis (Cheung et al., 2014a). The lysis of eukaryotic cells by PSMs, which occurs at micromolar concentrations, is likely mediated in a receptor-independent manner (Kretschmer et al., 2010), While the involvement of receptors cannot be formally excluded, the facts that PSMs are surfactant molecules, lyse artificial phospholipid vesicles (Duong et al., 2012; Laabei et al., 2014), and FPR2 is not involved in lysis (Kretschmer et al., 2010), speak for such a receptor-independent mechanism. Lytic activity is, however, heavily influenced by membrane composition (Laabei et al., 2014). It is believed to be crucial for PSM-mediated immune evasion and progression of staphylococcal pathogenesis. PSM-mec follows the characteristic cytolytic profile of some α-type PSMs and can lyse human erythrocytes and neutrophils at a comparatively moderate level that is similar to that exerted by δ-toxin (Queck et al., 2009).

THE *psm-mec* srRNA

The first clue for the presence of an srRNA within the *psm-mec* locus stemmed from experiments with MRSA strains transformed with a plasmid carrying 575 nucleotides amplified from SCC*mec* type II, termed the "F region" (Kaito et al., 2008). The authors reported that an open reading frame (ORF) transcribed in the opposite direction of the *psm-mec* gene, coined "*fudoh*," encoded a protein that controlled colony spreading (Kaito et al., 2008). It was later discovered from experiments using *S. aureus* isogenic *fudoh* point-deletion mutants that the phenotypic differences were in fact influenced by an srRNA (Kaito et al., 2011), which contains the *psm-mec* gene (Kaito et al., 2011, 2013), in a way similar to the genetic layout of δ-toxin, whose gene is found within the regulatory RNA of the *agr* system, RNAIII (Novick, 2003). Interestingly, our group reported that the *psm-mec* srRNA transcript length was 143 bp, 14 nucleotides shorter than that described by Kaito et al. (2013) (**Figure 1C**). Notably, in any case, the *psm-mec* srRNA only barely exceeds the ORF coding for PSM-mec. This contrasts with RNAIII, which is considerably longer than the δ-toxin gene, *hld*. Additionally, the *psm-mec* srRNA is estimated to have a half-life of approximately 20 min (Kaito et al., 2013), which is significantly shorter than the ∼45 min half-life described for RNAIII (Huntzinger et al., 2005). The only reported mechanism by which the *psm-mec* srRNA exerts it gene regulatory activity is through its interaction with the *agrA* transcript resulting in the overall decrease of AgrA activity (Kaito et al., 2013), which is entirely dependent on the first 60 nucleotides of the transcript (Kaito et al., 2013), where key anti-sense base-pair interactions occur (Cheung et al., 2014b). Because AgrA strictly regulates activity of the *psm*α and *psm*β promoters (Queck et al., 2008), many of the *psm-mec* srRNA-mediated phenotypes likely are mostly due to a repression of the production of the strongly cytolytic and pro-inflammatory PSMα peptides (**Table 1**). Kaito et al. initially observed up to eight-fold reduction in PSM expression when the *psm-mec* locus was over-expressed on a plasmid in methicillin-sensitive *S. aureus* strains or strains with SCC*mec* type IV (Kaito et al., 2011). However, the differences in PSM reduction were less dramatic, and often inconsistent, in isogenic *psm-mec* mutants naturally harboring SCC*mec* types II and III (Chatterjee et al., 2011), indicating a high degree of strain dependence regarding the role of the *psm-mec* srRNA in *S. aureus*.

THE IMPACT OF THE *psm-mec* srRNA ON BIOFILM FORMATION, COLONY SPREADING, AND EXPRESSION OF *S. aureus* VIRULENCE FACTORS

PSM peptides have a considerable impact on biofilm formation, which is based on their detergent-like properties (Vuong et al., 2000; Kong et al., 2006). PSMs cause the structuring and maturation of biofilms, which includes the formation of nutrient-transporting channels (Wang et al., 2011; Periasamy et al., 2012). PSMs also facilitate the detachment of biofilm clusters *in vitro* and *in vivo* (Wang et al., 2011; Periasamy et al., 2012; Otto, 2013b). It was shown that isogenic *agr* mutants (Vuong et al., 2000, 2004), total *psm* deletion mutants in all *psm* genes, and even single mutants in either the *psm*α, *psm*β, or *hld* loci (Wang et al., 2011; Periasamy et al., 2012) formed thicker biofilms compared to their wild type counterparts. Therefore, it was surprising to find that several isogenic *psm-mec S. aureus* mutants showed a reverse phenotype, albeit the change in biofilm formation was overall minor (Queck et al., 2009; Kaito et al., 2013). A phenotype of increased aggregation similar to that conferred by other PSMs (Dastgheyb et al., 2015) was only found in a strain in which the PSM-mec peptide was expressed in high relative amounts as compared to other PSMs (Queck et al., 2009). In most *psm-mec*-positive *S. aureus* strains, the regulatory effect of the *psm-mec* srRNA on the expression of other biofilm-dispersing

TABLE 1 | Activities of the PSM-mec peptide and the p*sm-mec* srRNA.

Virulence determinant within the *psm-mec* locus	Known *in vitro* activities in *S. aureus*
PSM-mec peptide	FPR2-mediated pro-inflammatory activity at nM concentrations: • Increased CD11b expression • Increased gp91phox expression • Increased IL-8 expression • Increased calcium flux • Increased chemotaxis Receptor-independent cytolytic activity at μM concentrations toward: • Erythrocytes • Neutrophils Peptide expression decreases oxacillin resistance[a]
psm-mec srRNA	Represses AgrA activity through direct interaction • Decreased PSM production[b] • Enhanced biofilm formation and aggregation[b] • Suppresses colony spreading • Up-regulates SpA expression[b]

[a] *Only tested in one background strain.*
[b] *Strain-specific phenotype.*

PSMs (Periasamy et al., 2012) appears to over-shadow the lack of the direct dispersion effect of the PSM-mec peptide in *psm-mec* mutants (Kaito et al., 2013). Thus, in most strains, the impact of the *psm-mec* locus on biofilm formation is opposite to that of other *psm* loci, although that effect is generally not very pronounced.

For many pathogenic bacteria, motility plays an important role in colonization and virulence (Cossart, 2002; Josenhans and Suerbaum, 2002; Krukonis and DiRita, 2003). Motility is commonly dependent on the expression of flagella and type IV pili (Kearns, 2010). Even though *S. aureus* has always been historically regarded as non-motile, as it does not have the genes for flagella or pili, *S. aureus* has repeatedly been shown to spread efficiently across the surface of soft agar (Kaito et al., 2011; Tsompanidou et al., 2012) and more recently, on fresh pork meat (Tsompanidou et al., 2013), through a PSM-dependent mechanism (Lin et al., 2016), an effect now known as "colony-spreading" (Kaito and Sekimizu, 2007).

Originally, the observations of colony spreading and several other phenotypes were made using plasmid-based expression of the *psm-mec* locus in MRSA strains that did not carry the *psm-mec* locus, such as *S. aureus* isolates that were either methicillin-sensitive or MRSA strains that have recently emerged in the community (Community-Associated MRSA, CA-MRSA (Lowy, 1998; DeLeo et al., 2010). CA-MRSA strains are genetically distinct and notably more aggressive than HA-MRSA strains, show enhanced production of virulence factors, and are capable of causing disease in otherwise healthy individuals outside public healthcare settings (Tacconelli et al., 2004; Diep and Otto, 2008; Li et al., 2009; DeLeo et al., 2010). Furthermore, CA-MRSA strains characteristically possess SCC*mec* types IV or V (Diep and Otto, 2008; DeLeo et al., 2010) and therefore lack the *psm-mec* locus. Interestingly, CA-MRSA strains were reported to have a greater capacity to spread on soft agar compared to HA-MRSA strains (Kaito et al., 2008) and the absence of the *psm-mec* locus from CA-MRSA strains was speculated to cause that phenotype. Indeed, CA-MRSA strains transformed with plasmids containing the *psm-mec* locus were impaired in colony spreading. Conversely, isogenic HA-MRSA *psm-mec* mutants showed enhanced colony spreading (Kaito et al., 2013). Moreover, the inability of synthetic PSM-mec to rescue the colony spreading phenotype in an *agr* deletion strain of *S. aureus* (Tsompanidou et al., 2013) indicated that this regulation is predominantly mediated by the *psm-mec* srRNA. However, other studies showed that colony spreading is positively regulated by *agr* (Tsompanidou et al., 2011) and mainly controlled by mechanisms independent of the *psm-mec* srRNA, which include the production of several staphylococcal virulence factors, such as PSMs (Omae et al., 2012; Tsompanidou et al., 2013) and teichoic acids (Kaito and Sekimizu, 2007). In contrast, colony spreading is suppressed in the presence of multiple staphylococcal cell surface proteins (Tsompanidou et al., 2012). Taken together, the molecular mechanisms behind colony spreading in *S. aureus* reveal a somewhat complex network involving different, and often opposing, signals that affect the final phenotypic outcome. Lastly, it remains unknown whether colony spreading on agar surface is a mechanism universally adopted by all staphylococci and to what extent it matters for the colonization of epithelial surfaces *in vivo*.

In an effort to investigate *psm-mec*-dependent regulation in the natural strain background without artificial plasmid expression-induced effects, our group used microarray profiling to compare the transcriptomes of wild-type strains compared with those of isogenic *psm-mec* mutants (Cheung et al., 2014b). Most strikingly, the primary conserved target of *psm-mec*-dependent gene regulation was *spa*, the gene coding for the immune evasion protein, surface protein A (SpA), which was independently confirmed (Kaito et al., 2011). We also found that the strength of *psm-mec* srRNA transcription affected SpA expression (Cheung et al., 2014b). The up-regulation of SpA by the *psm-mec* srRNA is especially interesting because it adds to a number of existing transcriptional regulators known to control *spa* (Gao and Stewart, 2004). The up-regulated expression of SpA may play an important but yet unidentified role in the manifestation of staphylococcal disease (Gomez et al., 2004; Cheng et al., 2009; Merino et al., 2009; Kim et al., 2011) in some *psm-mec*-harboring *S. aureus* strains.

THE IMPACT OF THE *psm-mec* LOCUS ON *S. aureus* VIRULENCE

Our group demonstrated that the ability of an isogenic *psm-mec S. aureus* mutant to form skin abscesses and cause sepsis in mouse models of infection was severely attenuated compared to the parent strain (Queck et al., 2009), indicating that the PSM-mec peptide contributed significantly to *S. aureus* pathogenesis. However, this phenotype could only be produced using an isogenic *psm-mec S. aureus* mutant whose parent strain produced more PSM-mec relative to other PSMs as compared to most *psm-mec*-harboring strains. Kaito et al. reported that the *psm-mec* srRNA contributes to virulence, but in a fashion opposite to that mediated by the PSM-mec peptide (Kaito et al., 2011, 2013). Furthermore, those authors proposed that the *psm-mec* srRNA was generally responsible for the differences in virulence between HA- and CA-MRSA strains (Kaito et al., 2013). They also speculated that point mutations in the *psm-mec* locus underlie increased virulence of a certain lineage, ST764, but this was not directly shown using isogenic mutants (Suzuki et al., 2016). However, a phenotype of increased virulence is not always observed in isogenic *psm-mec* HA-MRSA strains (Chatterjee et al., 2011), suggesting that the contribution of *psm-mec* srRNA toward *S. aureus* pathogenesis is strongly strain-dependent and cannot serve as a generally applicable explanation for differences in virulence between CA- and HA-MRSA strains.

THE ROLES OF THE PSM-mec PEPTIDE AND THE *psm-mec* REGULATORY RNA IN OTHER *Staphylococcus* SPECIES

In addition to *S. aureus*, the *psm-mec* locus is detected in many different methicillin-resistant *Staphylococcus* species (Monecke et al., 2012). However, PSM-mec expression levels have only been rigorously studied in *S. epidermidis* (Queck et al., 2009). Our

group showed that PSM-mec expression was only detected in methicillin-resistant *S. epidermidis* infection isolates, but never in methicillin-sensitive *S. epidermidis* strains (Queck et al., 2009). There is no amino acid or nucleotide sequence difference (100% identity) between the *S. aureus* and *S. epidermidis* PSM-mec peptide or srRNA, respectively, based on a comparison of strains *S. aureus* MRSA252 and *S. epidermidis* RP62A, and differences are also absent or very minor in other strains. This is in accordance with the notion of a relatively recent move of SCC*mec* from coagulase-negative species to *S. aureus*.

To date, only one study has studied the impact of the *psm-mec* locus on the phenotypes in *S. epidermidis* and *S. haemolyticus* (Ikuo et al., 2014). The results, which were based on *S. epidermidis* and *S. haemolyticus* strains without SCC*mec* transformed with plasmids harboring the *psm-mec* locus, showed a decrease in PSM production, as demonstrated in similar experiments with *S. aureus* (Kaito et al., 2011). Intriguingly, an increase of biofilm formation was only observed in experiments with *S. epidermidis*, as reported previously (Kaito et al., 2011), but not *S. haemolyticus*, suggesting that the impact of the *psm-mec* locus on virulence phenotypes among staphylococci is also highly strain-dependent. The role of PSM-mec in coagulase-negative staphylococci has not yet been directly investigated using isogenic deletion mutants.

CONCLUSION

It has become clear that the effects exerted by the *psm-mec* locus in *S. aureus* on virulence and virulence phenotypes such as biofilm formation are highly strain-dependent. They are also commonly rather moderate in extent compared to those conferred by other members of the PSM family. While great strides have been made to better understand the molecular underpinnings of gene regulatory *psm-mec*-dependent mechanisms in *S. aureus* pathogenesis, in the future the focus should be shifted toward the investigation of how the *psm-mec* locus affects the pathogenesis in other *Staphylococcus* species, especially *S. epidermidis* and *S. haemolyticus*, which, alongside *S. aureus*, are the most frequent and pathogenic agents of hospital-associated infections around the world. As it is a commonly accepted notion that SCC*mec* elements originate from coagulase-negative staphylococci (Otto, 2013c), such investigation is bound to shed light on the "original" role of the *psm-mec* locus in staphylococcal physiology, with the potential to also explain the contrasting roles of *psm-mec* in the *S. aureus* background.

AUTHOR CONTRIBUTIONS

LQ, JM, GC, and MO contributed to the drafting of the manuscript and approved the final version.

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Sub-MIC Tylosin Inhibits *Streptococcus suis* Biofilm Formation and Results in Differential Protein Expression

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Streptococcus suis (*S.*suis) is an important zoonotic pathogen that causes severe diseases in humans and pigs. Biofilms of *S. suis* can induce persistent infections that are difficult to treat. In this study, the effect of tylosin on biofilm formation of *S. suis* was investigated. 1/2 minimal inhibitory concentration (MIC) and 1/4 MIC of tylosin were shown to inhibit *S. suis* biofilm formation *in vitro*. By using the iTRAQ strategy, we compared the protein expression profiles of *S. suis* grown with sub-MIC tylosin treatment and with no treatment. A total of 1501 proteins were identified by iTRAQ. Ninety-six differentially expressed proteins were identified (Ratio $> \pm 1.5$, $p < 0.05$). Several metabolism proteins (such as phosphoglycerate kinase) and surface proteins (such as ABC transporter proteins) were found to be involved in biofilm formation. Our results indicated that *S. suis* metabolic regulation, cell surface proteins, and virulence proteins appear to be of importance in biofilm growth with sub-MIC tylosin treatment. Thus, our data revealed the rough regulation of biofilm formation that may provide a foundation for future research into mechanisms and targets.

Keywords: proteomics, *S. suis*, biofilms, tylosin, iTRAQ

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INTRODUCTION

Streptococcus suis (*S. suis*) is an important zoonotic pathogen that causes a wide range of diseases in pigs, including meningitis, septicaemia, pneumonia, endocarditis, and arthritis (Gottschalk et al., 2010). In addition, this pathogen is an emerging zoonotic agent and an important public health issue in East and Southeast Asia (Sriskandan and Slater, 2006; Gottschalk et al., 2007). During a single outbreak in China in 2005, more than 200 human cases of *S. suis* were reported, with a death total of 39 (Yang et al., 2006). Current studies show that *S. suis* can cause persistent infections by forming biofilms *in vivo* (Wang et al., 2011).

Biofilms are assemblages of microorganisms characterized by cells that are irreversibly attached to a substratum and embedded in a matrix of self-produced extracellular polymeric substances such as exopolysaccharides (EPS), proteins, nucleic acids and other substances; this type of sessile community-based existence is a critical characteristic for bacterial persistence (Davey and O'Toole, 2000). The physical and biological properties of the biofilm, such as slow growth and mechanical barrier, have a substantial role in the development of increased antimicrobial tolerance. Because the bacteria in chronic infections are aggregated and are in close proximity, genes coding for resistance

to antimicrobials can be passed horizontally from one bacterium to the another (Bjarnsholt et al., 2013). The bacteria in biofilms could be 1000-times more difficult to kill by antibiotics than the same organism growing planktonically (Gilbert et al., 1997). Therefore, the control of biofilms is understood to be crucial.

Apart from surgical intervention (when applicable), antibiotics are the main option for the treatment of biofilm infections (Bjarnsholt et al., 2013). Previous studies showed that macrolides successfully inhibited *Staphylococcus aureus* biofilm formation and reduced its virulence when used at sub-inhibitory concentrations (Fujimura et al., 2008). In addition, a sub-minimal inhibitory concentration of erythromycin can inhibit *S. suis* biofilm formation (Zhao et al., 2015). Tylosin, a macrolide antibiotic produced by *Streptomyces fradiae*, is widely used as a veterinary medicine. However, there is still not much effective research of sub-MIC tylosin inhibiting biofilm formation of *S. suis in vitro*.

At present, most of the available information regarding biofilm formation by drug intervention is based on transcriptomic analyses. However, a limitation of transcriptomic analysis for identifying biofilm-regulated gene network raised concerns among investigators. The method of proteomics is thought to be an essential complement to transcriptomic analysis for discovering key regulators of biofilm (Sauer, 2003). Different immunogenic components of planktonically grown *S. suis* proteins such as secreted or cell wall-associated proteins had been studied by using immunoproteomic assays (Zhang and Lu, 2007a,b; Geng et al., 2008; Zhang et al., 2008). Additionally, our lab found that quorum-sensing played a crucial role leading to biofilm formation through quantitative proteomic analysis of *S. suis* biofilm inhibited by sub-MIC erythromycin treatment *in vitro* (Zhao et al., 2015). However, there are no reports regarding the proteomic analysis of sub-MIC tylosin inhibiting biofilm formation of *S. suis in vitro*.

We identified several proteins in sub-MIC tylosin inhibiting biofilm formation of *S. suis* by using iTRAQ technology in this study. The findings from the present study may provide a theoretical foundation for therapy of *S. suis* biofilm infection and provide references for finding new potential therapeutic targets.

MATERIALS AND METHODS

Growth of *S. suis* Planktonic Cells

S. suis (ATCC 700794) was grown in Todd-Hewitt yeast Broth (THB; Summus Ltd., Harbin, Heilongjiang, China) for 16–18 h at 37°C with constant shaking for biofilm assays (Wang et al., 2011).

Observation by Scanning Electron Microscopy (SEM)

Mid-exponential growth phase cultures of *S. suis* ATCC 700794 were adjusted to an optical density of 0.1 at 600 nm (OD600). Then, 2 mL cultures were transferred to the wells of a 6-well microplate containing an 11 × 11 mm sterilized rough glass slide (Mosutech Co., Ltd., Shanghai, China) on the bottom. After culturing for 72 h at 37°C without shaking, the glass slide was removed with tweezers, and the biofilms on the rough glass slide were washed with sterile PBS. The remaining biofilms were fixed with fixative solution [4% (w/v) paraformaldehyde, 2.5% (w/v) glutaraldehyde, 2 mM $CaCl_2$ in 0.2 M cacodylate buffer, pH 7.2] for 6 h and washed three times with 0.1 M PBS 10 min each, then fixed in 2% osmium tetroxide containing 2 mM potassium ferrocyanide and 6% (w/v) sucrose in cacodylate buffer. The samples were dried, gold sputtered with an ion sputtering instrument (current 15 mA, 2 min) and observed using SEM (FEI Quanta, Netherland).

Effect of Tylosin on Biofilm Formation Determined by the TCP Assay

Mid-exponential growth phase cultures of *S. suis* were adjusted to 0.2 of OD600. Then, 100 μL of cultures were added to each wells of a 96-well microplate with equal volume of tylosin solution with the final concentrations of 1/2 MIC (0.25 μg/mL), 1/4 MIC (0.125 μg/mL), 1/8 MIC (0.0625 μg/mL), and 1/16 MIC (0.03125 μg/mL), respectively. In addition, a negative control (with THB alone) and a positive control (with bacteria alone) were also included. After incubation at 37°C for 72 h without shaking, the medium was removed by aspiration and the wells were washed three times with sterile physiological saline. The remaining attached bacteria were fixed with 200 μL of 99% methanol (Guoyao Ltd., China) per well, and the plates were emptied after 15 min and left to dry. Then, the plates were stained for 5 min with 200 μL of 2% crystal violet (Guoyao Ltd., China) per well. The excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 200 μL of 33% (v/v) glacial acetic acid (Guoyao Ltd., China) per well. The amount of released stain was quantified by measuring the absorbance at 570 nm with a microplate reader (DG5033A, Huadong Ltd., Nanjing, Jiangsu, China). The reported values are the means of three measurements. The experiments were performed in triplicate.

Colony Forming Unit (CFU) Enumeration

Overnight cultures of *S. suis* were adjusted to an OD 600 of 0.2. Then, the bacteria were inoculated into 96-well microtiter plate wells containing 200 μL of THB alone (untreated wells) or adding the tylosin solution with the final concentrations of 1/4 MIC (0.125 μ g/mL). In addition, a negative control (with THB alone) and a positive control (with bacteria alone) were also included. After incubation at 37°C for 72 h without shaking, the medium was removed by aspiration, and the wells were washed three times with sterile physiological saline. Biofilm cells were removed from wells by sonication for 5 min in 200 μL of THB. The cell suspensions ($n = 3$) underwent 10-fold dilutions in THB, and 100 μL of each dilution was spot plated onto THB soft-agar plates and incubated at 37°C for 24 h. All the experiments were performed in triplicate.

Preparation of Protein Extracts

For biofilm cultures, *S. suis* was grown in THB in 100 mm polystyrene petri dishes at 37°C for 24 h. Then, the supernatant was removed and the dishes were washed twice with Tris-HCl buffer (50 mM, pH 7.5). The biofilms were detached by scraping. After being sonicated for 5 min (Bransonic 220; Branson Consolidated Ultrasonic Pvt. Ltd., Australia), the cells were centrifuged at 12,000×g for 10 min at 4°C. Then, the cell

pellets were washed twice with Tris-HCl buffer (Wang et al., 2012).

Protein Digestion and iTRAQ Labeling

Protein digestion was performed according to the reported FASP procedure (Wisniewski et al., 2009). In brief, 200 μg of proteins at two different conditions (1/4 MIC of tylosin treated cells and nontreated cells) were added into 30 μL STD buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0) and ultrafiltered (Microcon units, 30 kD) with UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0). To block reduced cysteine residues, 100 μL 0.05 M iodoacetamide was added into UA buffer and incubated for 20 min in the dark. The filters were washed three times with 100 μL UA buffer and twice with 100 μL DS buffer (50 mM triethylammoniumbicarbonate at pH 8.5). Finally, the proteins were digested with 2 μg trypsin (Promega) in 40 μL DS buffer at 37°C for 16–18 h. Then, the resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density at 280 nm using an extinctions coefficient of 1.1 of 0.1% (g/l) solution calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins.

For the iTRAQ labeling, the peptides were labeled with the 8-plex iTRAQ reagent by following the manufacturer's instructions (Applied Biosystems). Each iTRAQ reagent was dissolved in 70 μL of ethanol and added to the respective peptide mixture. The peptides from the *S. suis* biofilms treated by tylosin were labeled with 115 isobaric reagent, and the peptides from the nontreated *S. suis* biofilms were labeled with 116 isobaric reagent. Then, the samples were multiplexed and vacuum dried. Three independent biological experiments were performed.

Peptide Fractionation with Strong Cation Exchange (SCX) Chromatography

SCX chromatography using the AKTA Purifier system (GE Healthcare) was used to fractionate the iTRAQ labeled peptides. After being reconstituted and acidified with 2 mL buffer A (10 mM KH_2PO_4 in 25% of ACN, pH 2.7), the peptides were loaded onto a PolySULFOETHYL 4.6 × 100 mm column (5 μm, 200 Å, PolyLC Inc., Maryland, U.S.A.). Then, the peptides were eluted at 1 ml/min with a gradient of 0–10% buffer B (500 mM KCl, 10 mM KH_2PO_4 in 25% of ACN, pH 2.7) for 2 min, 10–20% buffer B for 25 min, 20–45% buffer B for 5 min, and 50–100% buffer B for 5 min. The elution was monitored by absorbance at 214 nm, and the fractions were collected after every 1 min. The collected fractions (∼30 fractions) were combined into 10 pools and desalted on C18 Cartridges [Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 ml, Sigma]. Each pooled fraction was concentrated by vacuum centrifugation and reconstituted in 40 μl of 0.1% (v/v) trifluoroacetic acid and stored at −80°C for LC-MS/MS analysis.

Liquid Chromatography (LC) Electrospray Ionization (ESI) Tandem Ms (MS/MS) Analysis by Q Exactive

Experiments were performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Thermo Fisher Scientific). A sample (10 μL) of each fraction was injected for the nano LC-MS/MS analysis. The peptide mixture (5 μg) was loaded onto a C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at 250 nl/min controlled by IntelliFlow technology for 140 min. MS data were acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Determination of the target value was based on predictive Automatic Gain Control (pAGC). The dynamic exclusion duration was 60 s. Survey scans were acquired at a resolution of 70,000 at m/z 200, and the resolution for HCD spectra was set to 17,500 at m/z 200. The normalized collision energy was 30 eV, and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with the peptide recognition mode enabled.

Sequence Database Searching and Data Analysis

The MS/MS spectra were searched using the MASCOT engine (Matrix Science, London, UK; version 2.2) in the Proteome Discoverer 1.3 (Thermo Electron, San Jose, USA.) against the Uniprot *S. suis* fasta database (38,369 sequences, downloaded March 4th, 2013) and the decoy database. False discovery rates (FDR) were calculated by running all spectra against a decoy database using the MASCOT software. To identify proteins, the following options were used: Peptide mass tolerance = 20 ppm, MS/MS tolerance = 0.1 Da, Enzyme = Trypsin, Missed cleavage = 2, Fixed modification: Carbamidomethyl (C), iTRAQ 8plex (K), iTRAQ 8plex (N-term), Variable modification: Oxidation (M). The quantification was performed based on the peak intensities of the reporter ions in the MS/MS spectra. The ratio of label 115 and 116 represents the expression of proteins with the protein identification confidence of a 1% FDR (Unwin et al., 2010). The proteins were considered over expressed when the iTRAQ ratio was above 1.5 and under expressed when the iTRAQ ratio was lower than 0.67. The Proteome Discoverer tool was used to categorize the proteins detected by Gene Ontology (GO) annotation according to the cellular component, biological process and molecular function.

RESULTS

Effect of Tylosin against Biofilm Formation *In vitro* by the TCP Assay

We evaluated the action of tylosin on biofilm growth *in vitro*. The MIC against *S. suis* was 0.5 $\mu g \cdot mL^{-1}$. Tylosin at 1/2 MIC and 1/4 MIC caused a significantly higher reduction in the biofilm-forming ability of *S. suis* compared with positive control ($p < 0.05$). However, there was no pronounced effect for 1/16 MIC and 1/8 MIC of tylosin on biofilm formation of *S. suis* ($p > 0.05$; **Figure 1**).

Direct Observation of Biofilm Formation *In vitro* by Sem

SEM analysis was performed to observe the 1/4 MIC of tylosin treated cells and nontreated cells biofilm formation by *S. suis* under same growth conditions. As shown in **Figure 2A**, the surface of the glass slide is almost entirely covered by the aggregates and microcolonies of *S. suis* when growth was carried out in the culture medium without tylosin. However, when the culture medium was added to 1/4 MIC of tylosin, the biofilms were characterized by the presence of small clusters of cells interspersed amongst individual cells (**Figure 2B**). This result showed that the biofilm formation of *S. suis* was inhibited by the tylosin of 1/4 MIC *in vitro*.

Colony Forming Unit (CFU) Enumeration

To better assess action of tylosin on biofilm quantitatively, the CFUs of *S. suis* were counted. The viability of *S. suis* treated with 1/4 MIC of tylosin was different from the viability of untreated *S. suis*. The number of CFUs/mL in treated biofilms (5.3 $\log_{10}$ CFUs/mL) was significantly fewer than in nontreated biofilms (6.5 $\log_{10}$ CFUs/mL; $p < 0.05$). The number of CFUs/mL in treated biofilms (5.3 $\log_{10}$ CFUs/mL) was significantly fewer than in nontreated biofilms (6.5 $\log_{10}$ CFUs/mL; $p < 0.05$) (**Figure 3**). The findings demonstrated that 1/4 MIC of tylosin remained effective in decreasing the viability of *S. suis*.

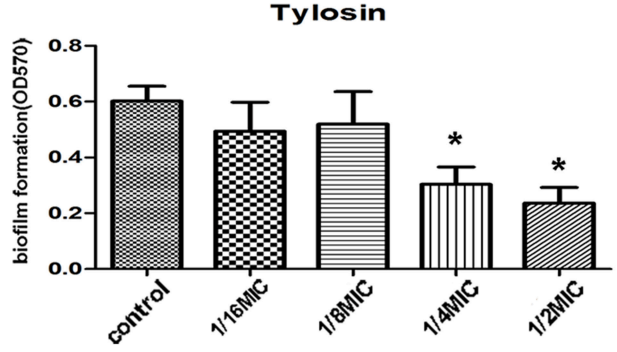

FIGURE 1 | Effect of tylosin at different concentrations on *S. suis* ATCC 700794 biofilm formation. The data are expressed as the means ± standard deviations. Significant decrease (*$p < 0.05$) compared with control biofilm formation of *S. suis in vitro*.

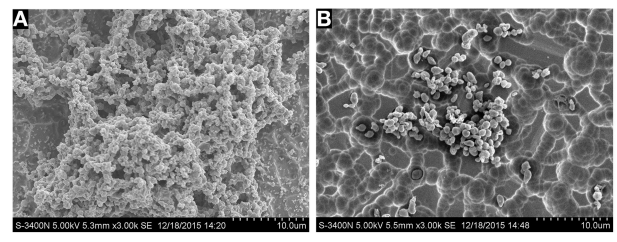

FIGURE 2 | (A) Biofilm formation of *S. suis* without tylosin. **(B)** Biofilm formation of *S. suis* with 1/4 MIC tylosin treatment.

Sub-MIC Tylosin Inhibits Biofilm Formation and Differentially Expressed Proteins by iTRAQ

A total of 1501 proteins were identified by iTRAQ. Detailed information is shown in the Supplementary File. A ratio of proteins with >1.5 or <0.67 ($p < 0.05$) was considered to be differentially expressed. Based on this criterion, 96 differentially expressed proteins were identified in 1/4 MIC tylosin treated cells and nontreated cells. Detailed information could is shown **Table 1**. These proteins were detected by GO annotation. Thirty-five proteins were deleted from the database. The remaining 61 proteins were classified into biological process, molecular function and cellular component (**Figure 4**). The results regarding the biological process were as follows: single-organism process (8, 13%), response to stimulus (4, 7%), localization (4, 7%), biological adhesion (1, 2%), cellular component organization or biogenesis (6, 10%), cellular process (25, 41%), biological regulation (3, 5%), metabolic process (30, 49%). The results regarding molecular function were as follows: binding (29, 48%), nucleic acid binding transcription factor activity (1, 2%), transporter activity (3, 5%), structural molecule activity (5, 8%), catalytic activity (25, 41%). The results regarding cellular component were as follows: organelle (6, 10%), virion (1, 2%), cell (13, 21%), extracellular region (1, 2%), membrane (6, 10%), macromolecular complex (8, 13%).

DISCUSSION

A TCP assay is based on the ability of bacteria to form biofilms on the bottom of tissue culture plates and is mainly used to identify the formation of bacterial biofilms (Mathur et al., 2006; Okajima et al., 2006; Presterl et al., 2007). Bacteria are grown in cell culture plates, and the quantity of a biofilm is detected by staining the wells with crystal violet according to the correlation between OD values and biofilm formation (Mathur et al., 2006).

To further confirm *S. suis* biofilm formation, the structure of the *S. suis* biofilm was identified by scanning electron microscopy

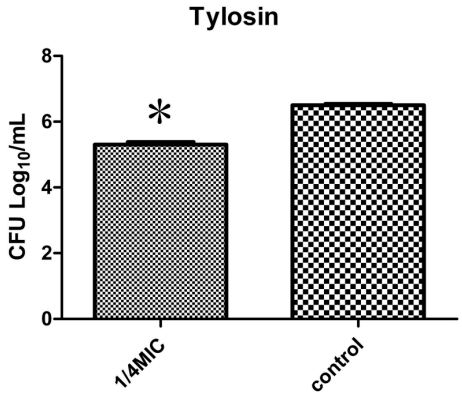

FIGURE 3 | Effect of 1/4 MIC of tylosin on *S. suis* ATCC 700794 biofilm formation by colony forming unit (CFU) enumeration. The data are expressed as the means ± standard deviations. Significant decrease (*$p <$ 0.05) compared with control biofilm formation of *S. suis in vitro*.

TABLE 1 | List of differentially expressed proteins in tylosin treated cells.

Accession	Description	Fold change[a]
G7SDH9	Putative uncharacterized protein	0.555777
A4W1G9	Phosphotransferase system cellobiose-specific component IIC	0.521547
G7SA24	Cold-shock DNA-binding domain protein	0.394546
F4EDP5	Putative uncharacterized protein	0.568016
R4NL31	MF3-like protein	0.63094
E9NQ29	CPS16V	0.452748
G5KZ73	NADH:flavin oxidoreductase/NADH oxidase family protein	0.459622
G7S4C2	Major membrane immunogen, membrane-anchored lipoprotein	0.643938
A8CUL3	Integrase	0.46773
G7SM99	Type I site-specific restriction-modification system, R (Restriction) subunit and related helicase	0.506277
G7SLJ0	Putative competence-damage inducible protein	0.564703
A4W1W3	F0F1-type ATP synthase, subunit a	0.586873
G7RZW0	Sugar ABC transporter permease	1.508325
G7S2T8	Alcohol dehydrogenase	0.610195
G7SDG9	Laminin binding protein	1.524593
R4NLJ6	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA	0.644177
G7S371	Fructose-bisphosphate aldolase	0.623705
M1UGE2	Uncharacterized protein	0.647168
A4VZM2	Cell cycle protein GpsB	0.631018
A4W2D2	Cation transport ATPase	1.676356
G5L1V6	50S ribosomal protein L9	0.6638821
G7SEP2	Putative uncharacterized protein	1.502566
A4VVZ3	tRNA dimethylallyltransferase	1.510883
G7S5B8	3-isopropylmalate dehydrogenase	1.513117
G7SA68	UDP-N-acetylmuramate–alanine ligase	0.656503
G5L098	Transcriptional regulator Spx	1.514636
E8UKC3	Uncharacterized protein	1.507837
G7SFU4	Putative uncharacterized protein	1.543591
A4VV99	DNA repair protein	1.545594
A4W2Z7	FMN-dependent NADH-azoreductase	1.527496
F4EC05	Putative uncharacterized protein	1.57743
C6GNL8	30S ribosomal protein S21	1.545471
K7ZNG8	DNA recombination/repair protein (Fragment)	1.558023
G7S3K5	ABC-type cobalt transport system, ATPase component	1.583854
G7SM56	Ribosomal RNA small subunit methyltransferase H	1.594936
A2VC24	Muramidase-released protein	1.579304
A4W3C1	Putative phosphotyrosine protein phosphatase	1.60975
G7S4U0	Putative uncharacterized protein	1.596783
G7SP92	Putative uncharacterized protein	1.658448
G7S7E3	Helicase	1.516925
M1VK55	Glycosyltransferase	1.662795
G7S3P9	Putative uncharacterized protein	1.673475
A4W106	Signal transduction histidine kinase	1.62492
B0M0G7	Phosphomethylpyrimidine kinase (Fragment)	1.633436
A5JSJ7	Putative uncharacterized protein	1.681661
G5L226	Type III restriction-modification system, restriction endonuclease subunit	1.697991
G7S7K4	Putative uncharacterized protein	1.645719
B9WY95	Peptidase M20	1.647536
G7S4T2	Branched-chain alpha-keto acid dehydrogenase subunit E2	1.673032
G5KX48	ABC-type uncharacterized transport system, permease component	1.705109
G7S178	Putative uncharacterized protein	1.774886
M1VDL7	ABC transporter permease protein	1.942183
G7S6Z3	Putative uncharacterized protein	1.956584
J7KIA5	Abortive infection bacteriophage resistance related protein	1.999106
G7SJZ7	Primosomal protein N'	1.720079
G7SHW7	Putative uncharacterized protein	2.001559
Q9EZW2	Elongation factor Tu (Fragment)	1.739971
A4VVJ5	Uncharacterized protein	2.141695
M1VJZ8	Zeta toxin	1.74309
G5L2V0	NADPH:quinone reductase and related Zn-dependent oxidoreductase	1.79706
R4NU43	Aromatic amino acid aminotransferase gamma @ N-acetyl-L,L-diaminopimelate aminotransferase	2.144605
A4W296	Uncharacterized protein	1.837367
G7SKQ0	CHAP domain containing protein	2.179882
B9WVD6	Putative uncharacterized protein (Fragment)	2.226792
G5L2N5	L-fucose isomerase	1.859887
B9WTA1	Putative uncharacterized protein	2.290848
B9WXU6	ABC transporter related protein	1.873566
G8DU82	Transposase	2.376768
G7S242	Putative ABC transporter	1.883471
A4VZ91	50S ribosomal protein L32	1.915877
G7SDX6	50S ribosomal protein L7/L12	1.918196
G5KZ86	Phosphatidylserine/phosphatidylglycerophosphate/ cardiolipin synthase-like protein	1.965488
G7SIQ5	Putative uncharacterized protein	2.451703
G7S7J2	Putative uncharacterized protein	2.481527
G7SPA1	Putative scaffolding protein	2.584249
G7S7A9	FAD-dependent pyridine nucleotide-disulfide oxidoreductase	1.970356
G7S8Q2	Peptidase M22 glycoprotease	1.978772
M1TIM7	Methylated DNA-protein cysteine methyltransferase	2.161583
G7SBK2	Elongation factor Ts	2.266517
M1VE47	Fic/DOC family protein	2.606274
M1UFP9	Thiamine-phosphate synthase	2.298028
G7S535	Fructose-6-phosphate aldolase	2.553516
G5L351	NsuB	2.694988
B9WUV5	Transcriptional regulator, DeoR family	2.65073
R4NW55	Plasmid replication protein Rep and AAA-class ATPase domain protein	2.779765
G7RZ18	Sortase-like protein	2.791504
S6B433	RecN protein (Fragment)	3.007243
G5KZR3	Glutathione S-transferase	3.262393
R4NLK5	SSU ribosomal protein S1p	3.46851
R4NWB6	Uncharacterized protein	8.306942

(Continued)

TABLE 1 | Continued.

Accession	Description	Fold change[a]
G5L259	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, putative	2.781098
D5AFN4	Xaa-Pro dipeptidyl-peptidase	3.095053
G7SMG3	Elongation factor G	3.667679
G7SD52	ABC superfamily ATP binding cassette transporter, membrane protein	3.201581
G5L1N9	Nucleoid DNA-binding protein	3.796473
G7S8P5	Phosphoglycerate kinase	11.34908

Fold change[a], the ratio of different expression level between Sub-MIC tylosin treated cells and nontreated cells.

(SEM). The biofilm examination relied heavily on SEM. Because of its high magnification, the biofilm microstructure is observed clearly. These findings further confirm that *S. suis* can form biofilms *in vitro*. Although these procedures have their advantages, the TCP procedure lacks specificity and sensitivity because crystal violet stains all of the components of the biofilm and could potentially stain non-biofilm material. More specific and sensitive techniques can be performed to assess each of the individual components. This method is good for screening purposes.

Tylosin is a frequently used drug for the treatment of *S. suis* infection. Upon treatment with this antimicrobial agent, *S. suis* is inevitably exposed to a sub-inhibitory level of the agent. Therefore, we studied its effect in sub-inhibitory concentrations on *S. suis* biofilm formation. Tylosin can inhibit *S. suis* biofilm formation at sub-inhibitory concentrations in a dose-dependent manner. In addition, the inhibition of biofilm formation varied among the antimicrobial agents. Numerous reports have showed biofilm formation in the presence of sub-inhibitory concentrations of antimicrobial agents (Dunne, 1990; Carsenti-Etesse et al., 1993; Rupp and Hamer, 1998; Rachid et al., 2000; Yang et al., 2015). Our results are in agreement with these previous findings. Our results showed a better activity of tylosin against *S. suis* biofilm formation. However, further studies should be conducted to confirm and clarify the relationship between the relative adherence-inhibiting properties of tylosin and their mechanisms. These results may provide information regarding the clinical use of antimicrobial agents against biofilm-forming bacteria.

Our data identified proteins related to biofilm growth that have previously been uncharacterized. iTRAQ analyses showed that the regulation of metabolism plays a key role during *S. suis* biofilm growth. First, the iTRAQ quantitative data revealed that carbohydrate metabolism may be particularly important during *S. suis* biofilm growth. It was reported that fructose bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase were significantly decreased in *Streptococcus pneumoniae* biofilms (Allan et al., 2014). In our study, there was a >two-fold increase of glyceraldehyde-3-phosphate dehydrogenase in the tylosin inhibiting biofilm formation. In addition, the levels of fructose bisphosphate aldolase (fold change: 0.62) were down-regulated. It was reported that phosphoglycerate kinase was up-regulated in *S. suis* biofilms compared with planktonic cells by comparative proteomic analysis (Wang et al., 2012). Similarly, biofilms of *Pseudomonas aeruginosa* (Sauer et al., 2002) and *Staphylococcus xylosus* (Planchon et al., 2009) exhibit up-regulated phosphoglycerate kinase. There was a >11-fold increase in the level of phosphoglycerate kinase in the tylosin inhibiting biofilm formation. Phosphoglycerate kinase is a glycolytic enzyme that functions in the conversion of glyceraldehyde 3-phosphate into 1, 3-diphosphoglycerate. Glycolysis may play a pivotal role during *S. suis* biofilm growth. Furthermore, histidine metabolism may play an important role in biofilm formation. Histidine kinase (fold change: 1.62) was up-regulated in the tylosin-inhibited biofilm formation. Compared with other amino acids, L-His had the strongest effect on biofilm induction (Cabral et al., 2011). Histidine metabolism is also found to be involved in biofilm formation and is confirmed by gene disruption (Cabral et al., 2011). Histidine kinase is an important signaling molecule in biofilm formation in gram-positive and negative bacteria (McLoon et al., 2011; Shemesh and Chai, 2013; Yang et al., 2014; Grau et al., 2015). In addition, several ABC transporter system proteins were significantly up-regulated in the tylosin inhibiting biofilm formation. This finding is consistent with the previous results of our laboratory showing that sub-MIC erythromycin inhibits *S. suis* biofilm formation (Zhao et al., 2015). The carbohydrate substrate selection and fermentation determine ABC transporter proteins were significantly up-regulated in tylosin-treated cells, suggesting that *S. suis* had the capability of metabolizing a wide range of carbohydrates during biofilm development (Hardy et al., 2001; Marion et al., 2011; Bidossi et al., 2012; Haertel et al., 2012). Moreover, ABC transporters are important because they regulate respiration and biofilm formation, which in turn affect the rate of electricity production and bioremediation (Selvaraj et al., 2014). Furthermore, in the mutants of a *pneumococcal* biofilm screen, ABC transporters were shown to be defective in colonization (Munoz-Elias et al., 2008).

Cell surface proteins play a crucial role in biofilms. Within the biofilm, bacterial cells are embedded in a self-produced extracellular matrix. This matrix protects bacteria against a number of environmental insults. However, the matrix also confines bacterial access to fresh nutrients. Thus, the verified increase in the expression of transmembrane channels appears to be an essential requirement for the entrance of important nutrient-containing fluid (Costerton et al., 1994). In addition to acting, as channels, porins may act as potential targets for adhesion to other cells and may mediate cell attachment through binding to the proteins released for biofilm formation (Dallo et al., 2010). The outer membrane proteins may mediate cell attachment through binding to the released proteins for biofilm formation. Membrane proteins such as OmpA mediate cell adhesion in *Acinetobacter baumannii* (Dallo et al., 2010). The key developmental stages of biofilm development have been reported to be adhesion to surfaces, aggregation of micro colonies, and further expansion of the microbial community. The putative involvement of many genes encoding large cell surface proteins is adhesion to the epithelium and biofilm formation (Pridmore et al., 2004; Walter et al., 2005; Frese et al., 2011). Pham (Pham et al., 2010) found increased expression of outer membrane proteins in the *Tannerella forsythia* biofilm cells by using quantitative non-gel-based proteomic techniques. In

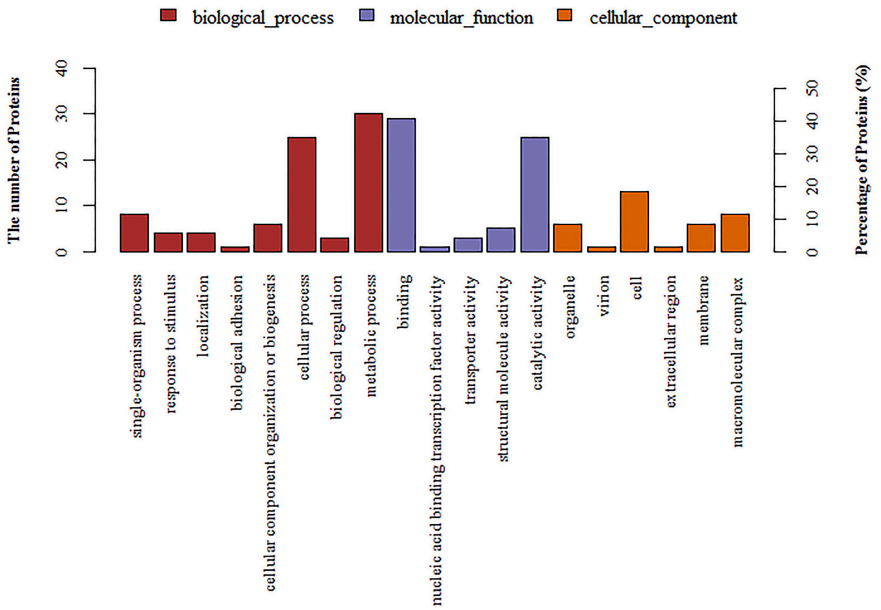

FIGURE 4 | Annotation of differentially regulated protein (ratio >1.5 or <0.67) functions by Gene Ontology (GO).

our study, the expression of membrane-anchored lipoprotein, phosphoglycerate kinase, sugar ABC transporter permease, ABC superfamily ATP binding cassette transporter membrane protein and L-fucose isomerase changed; these proteins belong to membrane proteins and cell-surface proteins and might be involved in some molecular functions including catalytic activity, motor activity, nucleotide binding, protein binding and transporter activity. For example, phosphoglycerate kinase is a *S. suis* surface protein that promotes cell adhesion and plays a key role in bacterial infection and invasion (Brassard et al., 2004; Wang and Lu, 2007). We predicted that these membrane proteins might affect the bacterial cell-cell interaction. Thus, membrane proteins might play a significant role in biofilm formation.

S. suis virulence proteins related to infection, persistence and competitive fitness were mostly down regulated when sub-MIC tylosin was used to inhibit biofilm formation. There was a >1.5-fold decrease in the level of NADH oxidase. NADH oxidase regulates competence, virulence, and pneumococcal persistence by its actions as an oxygen sensor, in detoxifying oxygen, and in increasing the efficiency of glucose breakdown (Auzat et al., 1999) and plays an important role in *pneumococcal* infection in animal models of pneumonia (Yu et al., 2001). NADH oxidase is encoded by *nox*. The virulence and persistence in mice of a blood isolate was attenuated by a *nox* insertion mutation (Auzat et al., 1999). Thus, this protein appears to be of major importance in the growth of biofilms.

CONCLUSIONS

Our study suggested that sub-MICs of tylosin could inhibit *S. suis* biofilm formation *in vitro*. We used a robust and reliable comparative proteomic technique (iTRAQ) to compare the abundances of proteins from 1/4 MIC of tylosin treated cells and nontreated cells. Finally, in sub-MIC tylosin inhibiting cells, we identified 96 differentially expressed proteins when the protein had a fold-change of more than a ratio >1.5 or <0.67 ($p < 0.05$). Our proteomic data suggested general changes in metabolism (such as phosphoglycerate kinase) and surface proteins (such as ABC transporter proteins) involved in biofilm formation. Overall, our results indicated that *S. suis* metabolic regulation, cell-surface proteins, and virulence proteins appear to be of importance in biofilm growth by sub-MIC tylosin treatment. Thus, our data revealed the rough regulation of biofilm formation that might potentially be utilized to manage biofilm infections of *S. suis*.

AUTHOR CONTRIBUTIONS

SW the design whole experiment. YL directed the completion of the experiment. YY, YZhao, HZ, JB, JC, YZhou, CW provided help during the experiment.

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SUPPLEMENTARY MATERIAL

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Absence of Protoheme IX Farnesyltransferase CtaB Causes Virulence Attenuation but Enhances Pigment Production and Persister Survival in MRSA

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The membrane protein CtaB in *S. aureus* is a protoheme IX farnesyltransferase involved in the synthesis of the heme containing terminal oxidases of bacterial respiratory chain. In this study, to assess the role of CtaB in *S. aureus* virulence, pigment production, and persister formation, we constructed a *ctaB* mutant in the methicillin-resistant *Staphylococcus aureus* (MRSA) strain USA500. We found that deletion of *ctaB* attenuated growth and virulence in mice but enhanced pigment production and formation of quinolone tolerant persister cells in stationary phase. RNA-seq analysis showed that deletion of *ctaB* caused decreased transcription of several virulence genes including RNAIII which is consistent with its virulence attenuation. In addition, transcription of 20 ribosomal genes and 24 genes involved in amino acid biosynthesis was significantly down-regulated in the *ctaB* knockout mutant compared with the parent strain. These findings suggest the importance of heme biosynthesis in virulence and persister formation of *S. aureus*.

Keywords: ***Staphylococcus aureus*, heme, antibiotics, pigment, virulence, persister formation**

INTRODUCTION

Staphylococcus aureus, named according to production of golden pigment, is an important human pathogen causing a variety of infection types including rampant skin and soft tissue infections, pneumonia, septicaemia, endocarditis, and central nervous system (CNS) infections. Methicillin-resistant *S. aureus* (MRSA) is notorious for its development of antibiotic resistance and expression of multiple virulence factors. (Li et al., 2012; Carrel et al., 2015).

S. aureus virulence factors are multifactorial and previous studies have been mainly focused on toxins (α-toxin, γ-toxin, Panton-Valentine leucocidin, exfoliative toxin and phenol-soluble modulins, etc.), surface proteins (FnbP, Bap, SasX, etc.) that help bind to host cells, facilitate

Abbreviations: MRSA, methicillin resistant *Staphylococcus aureus*; FPP, farnesyl diphosphate; SCV, small colony variants; MIC, minimum inhibitory concentration.

internalization and immune evasion. Staphyloxanthin, synthesized from farnesyl diphosphate (FPP) by CrtM and CrtN, is the main component of *S. aureus* golden pigment (Liu et al., 2005). Staphyloxanthin not only plays a protecting role in bacterial fitness, but enhances virulence and survive attack by neutrophils (Clauditz et al., 2006). In addition, global regulatory systems (Agr, SaeRS, SarA, etc.) govern different aspects of physiology and expression of virulence traits, maintaining a balance between fitness and virulence.

It was in *staphylococcus* that persisters were first described in Bigger (1944). Persisters represent a certain portion of a bacterial culture that is genetically identical but phenotypically resistant or tolerant to antibiotics and stresses. In the model organism *Escherichia coli*, much research reveals the mechanisms of persister formation, including involvement of toxin-antitoxin, protein degradation, energy production and DNA repair (Zhang, 2014). However, less understood are the mechanisms of *S. aureus* persister formation. The portion of persisters in *S. aureus* is so high that a hypothesis was proposed that unlike *E. coli*, all *S. aureus* cells in stationary phase are persisters (Keren et al., 2004). Subsequently, however, Lechner et al. proved that stationary phase cultures of *S. aureus* are also a mixture of regular and persister cells (Lechner et al., 2012).

Though the key mechanisms of *S. aureus* persister formation are poorly understood, progress has been made recently. It has been reported that biofilm formation (Lewis, 2001; Resch et al., 2006) and small colony variants (SCV; Lechner et al., 2012) are two key features involving *S. aureus* persister formation, probably because the cells in biofilms and SCV cells have a different profile of gene expression, which makes them more readily to form persisters. Glycerol uptake has been reported to play a role in persister formation. Mutation in the glycerol transporter encoding gene *glpF* caused defective survival of *S. aureus* to ampicillin and norfloxacin (Han et al., 2014). A point mutation of the inorganic phosphate transporter gene *pitA* enhanced tolerance to daptomycin (Mechler et al., 2015). Mutations in purine biosynthesis genes (*purB, purF, purH, purM,*) amino acid, lipid, carbohydrate metabolism, and energy production genes efflux etc. were found to cause decreased persister formation in recent transposon mutant library screens (Yee et al., 2015; Wang et al., 2015).

Heme synthesis is an important pathway in Gram positive bacteria and provide substrate to production of terminal oxidases (Mogi et al., 1994). Within vertebrates *S. aureus* fulfills its requirement of iron by uptaking heme-iron from transferrin or heme or hemoglobin with its several transporters including StrA, StrB, IsdA, and IsdE, etc. (Drabkin, 1951; Mazmanian et al., 2003; Liu et al., 2008; Mason and Skaar, 2009). However, in an environment without heme-iron, *S. aureus* has to synthesize heme A with a complex pathway starting from glutamate (Hammer et al., 2016). CtaB and CtaA catalyzes the last two steps of the process. CtaB is a heme O synthase (protoheme IX farnesyltransferase) and while CtaA is an integral membrane protein that converts heme O to heme A (Svensson et al., 1993; Svensson and Hederstedt, 1994; Clements et al., 1999). Heme A is essential for functional expression of the terminal oxidases. Among terminal oxidases synthesized with heme A, cytochrome aa 3 are quinol oxidases (QoxA, QoxB, etc.) and cytochrome caa 3 is a cytochrome c oxidase.

Though heme synthesis mainly contributes to the pathway of synthesis of terminal oxidases that mediate bacterial respiration, it has also been reported to participate in fitness and virulence of *S. aureus*. For example, CtaA was found to be required for starvation survival and recovery from glucose starvation (Clements et al., 1999). A correlation between heme production and pigment production was reported by Lan et al., as depletion of CtaA and QoxB both enhanced pigment production, while attenuating hemolytic activity and virulence (Lan et al., 2010). However, no study has been done to address the specific effects of *ctaB* mutation on the heme-to-respiratory chain pathway and associated phenotypic changes. In this study, we created a CtaB deletion mutant of *S. aureus* and found associations of CtaB with heme synthesis, pigment production as well as persister cell formation. In addition, we performed a transcriptome analysis to provide new insights into the basis of the above associations.

MATERIAL AND METHODS

Bacterial Strains, Growth, and Chemical Reagents

S. aureus USA500 (Diep et al., 2006) was used for construction of gene knockout and complementation strains. *E. coli* DC10B (Monk et al., 2012) was used for shuttle plasmid construction. Luria Broth medium was composed of 1% tryptone (Oxoid), 0.5% yeast extract (Oxoid) and 0.5% NaCl; BM (B-Medium) was composed of 1% tryptone, 0.5% yeast extract, 0.5% glucose, 0.1% K_2HPO_4 and 0.5% NaCl; BM and TSB (Tryptic soy broth, Oxoid) were used for *S. aureus* cultivation. Bacterial strains were inoculated in BM, and their growth rate at 37°C was monitored by measuring the OD values at 600 nm. Anhydrotetracycline (ATc) was used for induction of *secY* antisense RNA during gene knockout. Antibiotics were added to medium at the following concentrations: chloramphenicol, 10 μg/ml; ampicillin, 100 μg/ml, levofloxacin, 50 μg/ml.

Construction of Plasmids for Homologous Recombination and Complementary Strains

We constructed plasmid pMX10 by replacing *ccdB* element with multiple cloning sites in pKOR1 (Bae and Schneewind, 2006) and used it for construction of gene knock out strains. Primers pMX10-f and pMX10-r were mixed equally to a final concentration of 100 uM, incubated at 72°C for 20 min and slowly cooled to 4°C. The resulting dimers were digested with BamHI and KpnI and ligated to pKOR1 backbone digested with the same restriction enzymes. To construct *ΔctaB* in USA500, the upstream (us) fragment (about 1000 bp) at the upstream of *ctaB* gene of USA500 strain was amplified with primer ctaB-uf and ctaB-ur, while the downstream (ds) fragment with primers ctaB-df and ctaB-dr. The two fragments were then used as templates for fusion PCR with primer ctaB-uf and ctaB-dr. The final PCR product was digested with KpnI and MluI and then ligated into pMX10. The recombinant plasmids was transformed into

USA500 by electroporation and mutants were selected according to the method reported by Bae et al. (Bae and Schneewind, 2006). To construct the complementation strain *ΔctaB::pRBctaB*, a fragment containing the promoter region and coding sequence of *ctaB* gene was amplified with primers cp-ctaB-f and cp-ctaB-r. The PCR product was digested with EcoRI and BamHI and then ligated into plasmid PRB473. The resulting plasmid was transformed into the *ΔctaB* mutant via electroporation. The sequences of primers are listed in **Table 1**.

Detection of Pigment Production and Hemolytic Activity

To compare pigment production, USA500 and USA500ΔctaB were dropped onto TSA plates and USA500ΔctaB with pRB473 or pRBctaB on TSA plates with 10 μg/ml chloramphenicol. The plates were incubated at 37°C for 24 h and pictured. For quantitate assay of pigment production, the same strains were cultured in TSB at 37°C for 24 h. For each sample, pigment was extracted with methanol and detected with a parameter (GeneSpec III, Hitachi, Japan), following a previously reported protocol (Morikawa et al., 2001). For hemolytic activity determination, the strains were analyzed by growing the strains on 5% sheep blood agar at 37°C for 48 h. The result represents three independent experiments.

Mouse Infection

The mouse virulence test was performed on Balb/C mice. USA500 and the *ΔctaB* mutant strains were cultured for 18 h and 1 ml of the each culture was mixed with 2% Cytodex-1 beads by 1:1. The mice were randomized into two groups (5 mice/group). Each mouse was challenged with 200 μl bacterial mixture (each containing approximately 2×10^5 bacterial cells) via injection under skins on the back. After 48 h, the mice were sacrificed and the abscess under skin was homogenized in 2 ml PBS. The samples were diluted and plated on TSA plates at 37°C for 18 h. CFU counting was performed and a Student's *t*-test was used for statistical analysis using Microsoft Excel.

Animal studies on mice were performed according to relevant national and international guidelines (the Regulations for the Administration of Affairs Concerning Experimental Animals, China) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Medical College, Fudan University (IACUC Animal Project Number: 20110630). Standard operation procedures were followed to carry out animal experiments in bio-safety level 2 labs.

Susceptibility Testing

The MIC of each antimicrobial compound was determined in triplicate by a conventional broth microdilution technique in TSB medium, following the protocol previously published (Andrews, 2001) and the CLSI guidelines. The MIC was defined as the lowest antibiotic concentration that inhibited visible bacterial growth (also according to OD600 measurements) after 24 h of incubation at 37°C.

TABLE 1 | Primers used in this study.

Primers	Sequence 5′-3′	Purpose
pMX10-f	GGGGTACCGCTAGCCGGCCGG GGCCCACGCGTGAATTCCG	Construction of pMX10
pMX10-r	CGGAATTCACGCGTGGGCCCC GGCCGGCTAGCGGTACCCC	
ctaB-uf	GGGGTACCGCTGTATAACCAT AATGAACAGTACG	Construction of *ΔctaB*
ctaB-ur	CATCCTAACTTAATTAATATC CCCCTCCTTAAATTTGTTC	
ctaB-df	AATTTAAGGAGGGGGATTATTAAT TAAGTTAGGATGAAAAATATGGG	
ctaB-dr	CGACGCGTAGAAGTAAGCACTT TAATATCTTTACC	
cp-ctaB-f	CGGAATTCAAAAAGAACTTA ATCGTAATGATTTTTTTATTG	Construction of *ΔctaB::pRBctaB*
cp-ctaB-r	CGGGATCCCTTAATTAATCT AGATCAAAGTAAGTAATGAAAC	
RThld-f	CACTGTGTCGATAATCCATT	Real-time PCR
RThld-r	ATTAAGGAAGGAGTGATTTCAAT	
RTesaB-f	ACTTAGCAGTACCAGCATAT	
RTesaB-r	AATATCTCCATCAGCGATTTG	
RTset18-f	CAGAGCGATTAGCAATGATAA	
RTset18-r	GCGTTCTTGTCTTGTGTTA	
RThtrA-f	TGTGCTATTGAACGATAACG	
RThtrA-r	CTTGCTCTGCTTGATAACTC	
RTarcB2-f	TGAACCTGATGAAGTATGGA	
RTarcB2-r	TGGAAAGATGGTAAGCAATG	
RTsdhA2-f	CAGCAGATTTAGCATTAGCA	
RTsdhA2-r	TACGACCAACCTTATCCATT	
RTnrdE-f	CGATGGTATGGCTATTCCTA	
RTnrdE-r	CGATTGGCATTACAGAACTT	
RTpyrF-f	TAGATGGCGTTGTTTGTTC	
RTpyrF-r	GTAATACGGTGTTGGTCATT	
RTrpmC-f	TTAGAGACTTAACCACTTCAGA	
RTrpmC-r	CTTTCACGAGCAACAGTTT	
RTagrD-f	AACATTGGTAACATCGCAG	
RTagrD-r	GTGTTAATTCTTTTGGTACTTCA	
RTdltA-f	TGGTTCATTCAAGGTCGTA	
RTdltA-r	GCATTGTCCGTAACTTCAG	
RTrrs1-f	GTGCTACAATGGACAATACAA	
RTrrs1-r	ACTACAATCCGAACTGAGAA	

Persister Assay

To determine the number of persister cells in exponential phase, cells were grown overnight in 4 ml and were inoculated to 10 ml of fresh medium to an initial OD600 of 0.05. Cultures were shaken for 1.5–2 h (for normally growing cells), until an OD600 of approximately 0.5 was reached. To determine the number of persister cells in stationary phase, overnight cultures (16 or 24 h) were used without dilution.

For heat stress assay, stationary phase cultures were incubated at 57°C for up to 3 h. For oxidative stress assay, stationary phase cultures were diluted by 1:100 in TSB that contained 50 mM hydrogen peroxide (H_2O_2) for 4 h.

For starvation stress assay, stationary phase cultures were centrifuged, washed and resuspended in 3% NaCl. The survival of bacteria was determined by CFU counting at each hour. All stress assays were conducted using at least three biological replicates.

For antibiotic exposure, 2 ml of the overnight or the exponential phase cultures was transferred to 14 ml culture tubes (Greiner), antimicrobials were added at 100-fold MIC as indicated and the cultures were shaken for 12 h, or for 7 days during long-term experiments. For CFU determination, 100 μl was taken before and during antimicrobial challenge on an hourly basis during the first 8 h and after 24 h, or after 1, 2, 3, 5, 6 days during long-term experiments. Cells were washed in PBS and spotted as 10 μl aliquots of serial dilutions onto TSA plates. Colonies were counted after incubation for 24 h at 37°C. The lower limit of quantification was 100 CFU/ ml. All time-kill experiments were conducted using at least three biological replicates.

RNA Isolation, mRNA Enrichment and Sequencing

S. aureus USA500 parent strain and *ΔctaB* mutant were cultured for 6 or 24 h as log phase and stationary phase cultures. The cultures were divided into 3 aliquots and treated with RNAprotect (Qiagen) and frozen at −80°C. Total RNA was extracted from bacterial cells using the RNeasy Mini kit (Qiagen) as described (Atshan et al., 2012). The quality of RNA samples was examined with Bioanalyzer 2100 RNA-6000 Nano Kit. To remove 16S and 23S rRNAs, 10 μg of high-quality total RNA was processed using the Ribo-Zero™ Gold Kit before precipitating with ethanol and resuspending into 25 μL of nuclease-free water. The cDNA libraries with 150- to 250-bp multiplexed cDNA were generated from the enriched mRNA samples using the TruSeq Illumina kit (Illumina, San Diego, CA), following instructions from the manufacturer.

Sequencing was performed with HiSeq2500 (Illumina). The Cufflinks suite of tools were used to assess and quantify the total number of reads. With the program Cuffdiff as part of the suite, transcripts were quantified by assessing the total number of reads for the entire transcript. Briefly, reads were mapped to annotated coding sequences (CDSs) from genome of *S. aureus* USA300 TCH1516 strain since USA500 is the progenitor of USA300. The samples to be compared were evaluated for variance and tested for differential expression. Reads' *P*-values were determined, and significance was assessed by conducting Benjamini-Hochberg correction for multiple testing. The transcript sequencing data were submitted to the NCBI Sequence Read Archive, available for access under a RUN number RSS3919726.

Quantitative Real-Time PCR

For quantitative Real-time PCR, the same RNA samples were taken from that used for RNA-seq. After reverse transcription with cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), qRT-PCR was performed using SYBR Green PCR reagents (Takara Biotechnology) to determine the relative expression levels of the target genes with gene-specific primers listed in **Table 1**. The housekeeping gene *rrs1* (16s RNA) was used as an endogenous control. All qRT-PCR experiments were carried out in triplicate with independent RNA samples and the $2^{-\Delta\Delta CT}$ method was performed for analysis of relative gene expression data (Livak and Schmittgen, 2001).

Statistics

The significance of experimental differences in pigment production, hemolytic activity, survival *in vivo* and persister assay was evaluated by unpaired Student's *t*-test.

RESULTS

Construction and Properties of the *S. aureus ctaB* Deletion mutant *ΔctaB*

To investigate the functions of CtaB, we constructed a *ctaB* deletion mutant, *ΔctaB*, via homologous recombination, as well as made a complemented strain *ΔctaB::pRBctaB* by inserting *ctaB* with its own promoter into plasmid pRB473. When grown in TSB, the *ΔctaB* mutant showed a slight growth defect, compared with the parent strain USA500 (**Figure 1A**). The *ΔctaB* mutant displayed enhanced golden pigment production when grown on TSA for 24 h compared with the control strain (**Figure 1B**), and complementation of the *ΔctaB* mutant reduced pigment production to normal levels (**Figure 1B**). Quantification of pigment production by extracting carotenoid products confirmed that CtaB depletion afforded enhanced pigmentation than USA500 strain (**Figure 1C**).

CtaB Affects Hemolytic Activity and Survival *In vivo*

Hemolytic ability is an important aspect of *S. aureus* virulence (Wang and Muir, 2016). We analyzed the level of bacterial growth and hemolysis of the *ΔctaB* mutant on sheep blood agar plates. While all strains showed similar sized colonies, deletion of *ctaB* generated a strain with reduced hemolytic activity, which could be restored by complementation of the *ΔctaB* mutant with the wild type *ctaB* gene (**Figure 2A**).

Having observed that the *ΔctaB* mutant enhanced pigment production but reduced hemolytic activity, we wondered whether *ΔctaB* mutant would affect virulence *in vivo*. Skin is one of the most frequently targeted sites for *S. aureus* infection (Liu, 2009). To determine whether CtaB is associated with virulence during *S. aureus* infection, we compared the *ΔctaB* mutant and the parent strain in a mouse model of skin abscess. Colony counting of bacteria from mouse skin lesions showed that inactivation of *ctaB* attenuated bacterial survival *in vivo*. After 24 h of infection, the CFU counting of USA500 increased from $6.18 \pm 0.46E + 6$ to $6.79 \pm 1.02E + 6$. Within contrast, the *ΔctaB* mutant survived less well with a decrease of CFU, from $4.74 \pm 0.57E + 6$ to $2.96 \pm 1.3E + 6$. (**Figure 2B**).

CtaB is Involved in Persister Cell Formation under Stress and Antibiotic Treatment

To determine if CtaB is involved in persister formation or survival, we subjected stationary cultures of USA500, *ΔctaB* and *ΔctaB::pRBctaB* under stress conditions including heat, oxidative stress, and starvation. The CtaB mutation attenuated the ability of

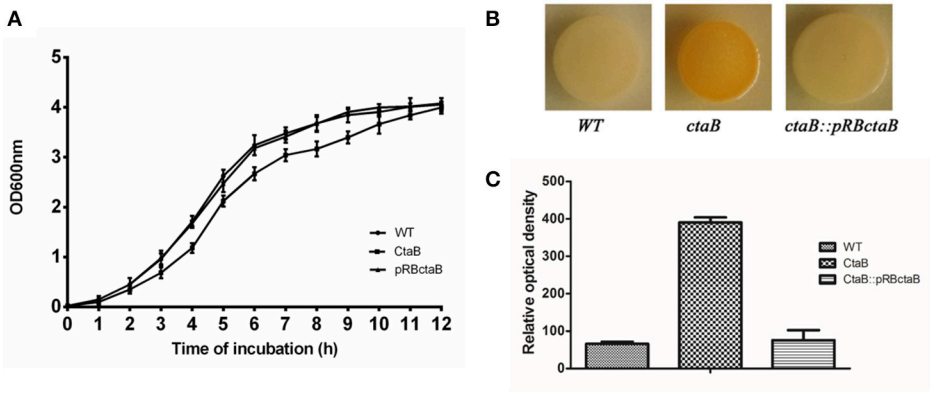

FIGURE 1 | (A) Comparison of the growth rate of USA500 and the *ΔctaB* mutant. A saturated overnight culture of each strain was inoculated in a 12 ml tube and cultured at 37°C. Cultures were monitored by measuring absorption at OD600 each hour. **(B)** Pigmentation display of *S. aureus* strains grown on TSA plates at 37°C for 24 h. **(C)** Measurement of the golden pigment of different strains by methanol extraction. The relative optical density units were detected at 465 nm and normalized to the USA500 strain, which was set at 100. Results are means with standard error (error bars) of three independent experiments.

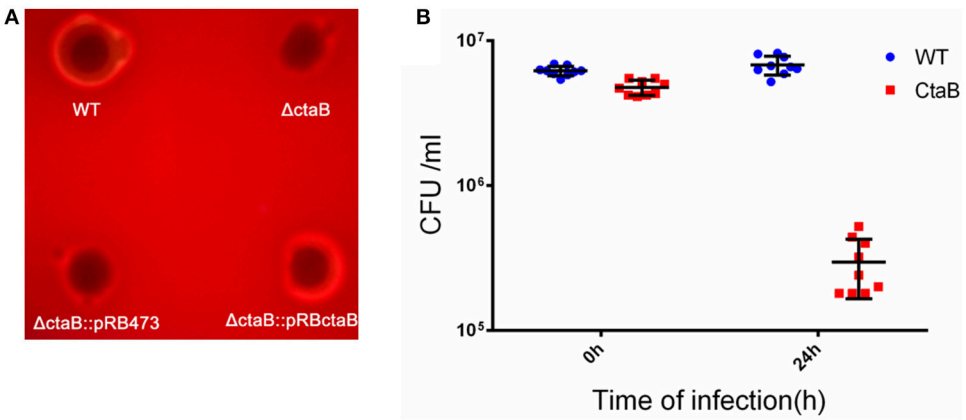

FIGURE 2 | (A) Hemolytic activity assay. Overnight cultures of USA500, *ΔctaB* mutant and complemented strains were spotted (20 μl) on sheep blood agar plates and grown at 37°C for 24 h. The result is representative of triplicate experiments. **(B)** Survival of *S. aureus* strains in a Balb/C challenged by subcutaneous injection. Comparison of CFU counts was performed using the Student's *t*-test. Results are means with standard error (errors bars).

S. aureus to survive starvation in 3% NaCl, which provides similar osmotic pressure as TSB, and the impact was reversed by gene complementation (**Figure 3A**). However, CtaB knockout did not affect survival of *S. aureus* under treatment with heat or H_2O_2 (data not shown).

Before persister assay, we measured the antibiotic sensitivity of *ctaB* mutant and found no difference in MIC tests for multiple antibiotics (data not shown). Challenging the stationary phase cultures of USA500, *ΔctaB* and *ΔctaB::pRBctaB* with 100 X MIC ciprofloxacin or levofloxacin yielded disparate killing curves. As shown in **Figures (3B,C)**, the surviving ratios of *ΔctaB* were similar with that of USA500 in the first 3 days, but became higher than the control strain in day four and day five. Meanwhile, complementation with plasmid pRBctaB but not pRB473 partially reversed the augmentation of persister formation caused by deletion of *ctaB* in the last 2 days of treatment. We also tested other antibiotics such as vancomycin, rifampicin, streptomycin, tobramycin, and gentamycin at 100X MIC concentration but found no significant difference in persister formation between the *ΔctaB* mutant and the parent strain from either exponential phase or stationary phase (data not shown).

RNA-seq Analysis of the *ΔctaB* Mutant Compared with its Parent Strain USA500

The above results indicate an intriguing and paradoxical role of CtaB in persistence and virulence of *S. aureus*, as its deletion attenuated virulence and survival in 3% NaCl while increasing persister numbers for quinolone antibiotics. To gain insights into the role of CtaB in altered *S. aureus* virulence and persistence, we performed RNA-seq analysis of USA500 and *ΔctaB* mutant grown for 6 h (log phase) or 24 h (stationary phase) in TSB medium. Based on the results of read counts of all annotated

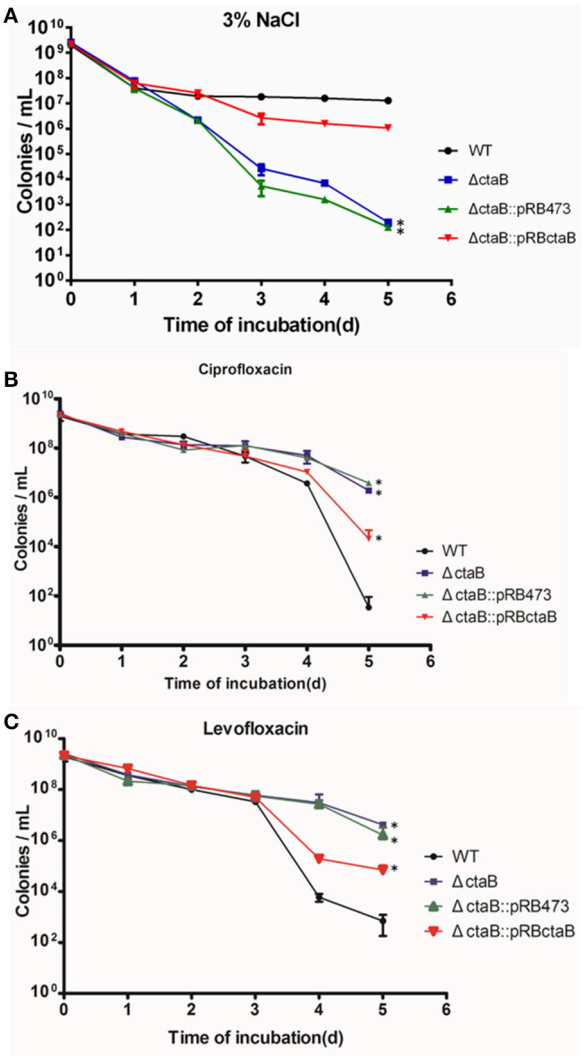

FIGURE 3 | Time dependent killing of *S. aureus* stationary phase bacteria. (A) Effects of starvation (3% NaCl) on survival kinetics of *S. aureus* USA500, *ΔctaB* mutant and complemented strain. Results are representative of three independent experiments. **(B,C)** Persister assay with antibiotics. Strains were treated with **(B)** 20 μg/ml ciprofloxacin or **(C)** 50 μg/ml levofloxacin for 6 days. The limit of detection was 100 CFU/ml throughout all killing experiments. Results are representative of three independent experiments.

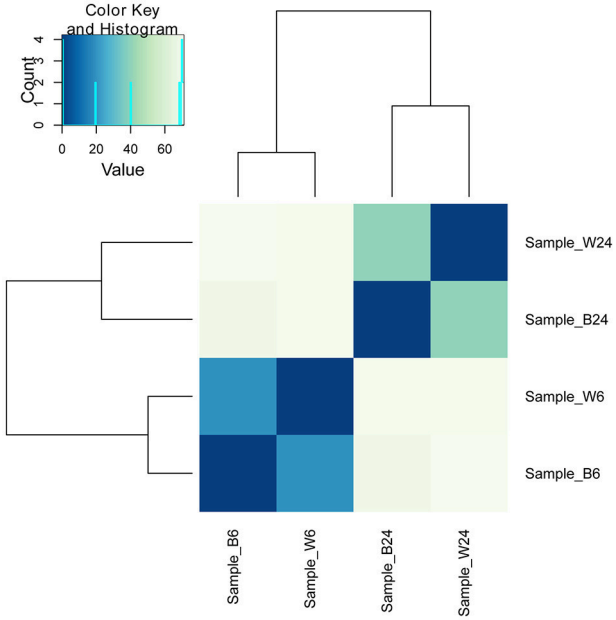

FIGURE 4 | Clustering of 4 RNA-seq samples. Sample_W6, USA500 grown to 6 h; Sample_B6, USA500ΔctaB grown to 6 h; Sample_W24, USA500 grown to 24 h; Sample_B24, USA500ΔctaB grown to 24 h. Heatmap shows the Euclidean distances between the samples as calculated from the variance-stabilizing transformation of the count data.

genes, a total of 4 RNA-seq samples were clustered without supervision (**Figure 4**). The results indicated that the effect of CtaB knockout on the bacterial transcriptome at 24 h were more apparent than that at 6 h (**Figures 5A,B**).

In log phase cultures, we found 18 genes with significant changes in transcription (cutoff > 2-fold) and *p*-values less than 0.05 between *ΔctaB* mutant and the parent strain (**Table 2**). Most strikingly, the virulence gene *hld* was significantly down regulated (0.35, $p = 8.47E\text{-}05$) in the *ΔctaB* mutant. In *S. aureus*, gene *hld* is located inside the coding sequence for small regulatory RNA RNAIII which regulates the expression of many *S. aureus* genes encoding exoproteins and cell-wall-associated proteins. The data indicated that deletion of CtaB could attenuate expression of various virulence genes regulated by RNAIII in log phase. Interestingly, CtaB deletion did not affect expression of the Agr system (encoded by *agrB*, *agrD*, *agrC*, and *agrA*), which is the well-known upstream regulator of RNAIII and a virulence factor. The virulence gene *esaB* (Burts et al., 2008; Anderson et al., 2011)was also down regulated. Meanwhile, the virulence genes *set18* and *set19* were up regulated in the *ΔctaB* mutant. Two hemin ABC transporter super family genes *htrB* and *htrA* were significantly up regulated, as a consequence of lack of heme caused by deletion of CtaB (**Table 2**).

For the transcripts at 24 h, 119 genes showed significant changes between *ΔctaB* mutant and the parent strain (**Table 3**), indicating that CtaB has a major impact on stationary phase *S. aureus*. The proposed pathways that these genes participate in were analyzed (**Figure 6**). Nineteen genes encoding ribosome proteins were strongly down regulated, as well as nine genes involved in biosynthesis of Aminoacyl-tRNA. Genes involved in arginine, proline, cysteine, methionine, glycine, serine, threonine, lysine, phenylalanine, tyrosine, tryptophan, valine, leucine, and isoleucine were down regulated in the CtaB mutant. Expression of several ABC transporters was also up regulated, but other transporters such as OppA1, OppB3, OppC1, OppD1, and OppF1, were down regulated. Twenty two genes that encode factors for amino acid metabolism showed difference in expression. Expression of two genes (*arcB2* and *arcC1*) involved in arginine metabolism was up regulated while the others were down regulated. The deletion of CtaB also down regulated genes from pathways involved in purine

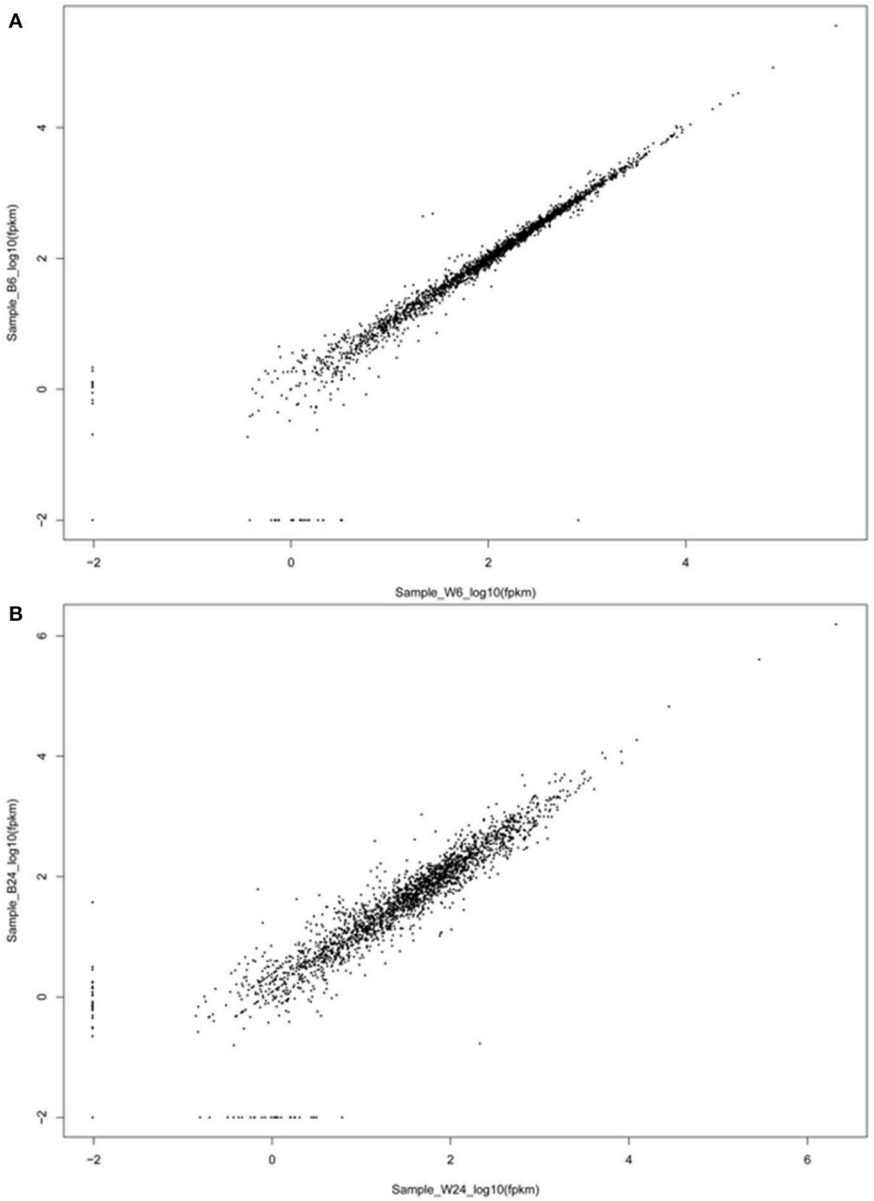

FIGURE 5 | Scatter plot of the expression levels of pairs of adjacent genes. The expression levels of two genes located within the same transcript of log phase bacteria **(A)** and stationary phase bacteria **(B)** are plotted in $\log_{10}$ scale.

metabolism, pyrimidine metabolism, and fatty acid biosynthesis. Though out of 17 genes associated with *S. aureus* infection 12 were up regulated, genes in the *dlt* operon (*dltA*, *dltB*, *dltC,* and *dltD*) were significantly down regulated (Collins et al., 2002). The CtaB deletion also induced expression of genes of five two-component systems, including PhoPR, LgrAB, SaeRS, and LytSR, indicating that these systems might play a role when *S. aureus* is confronted with lack of heme biosynthesis (**Table 3**).

Quantitative Real-time PCR was performed to validate the RNA-seq results. Genes were chosen from the list of genes with significant changes of transcription, favoring those associated with virulence and protein production but had a p-value < 0.05. All showed similar fold change with those from the RNA-seq results (**Figure 7**).

DISCUSSION

The aim of this study is to address the role of CtaB in pigment production, virulence and persister formation in *S. aureus*. We found that deletion of *ctaB* attenuated survival under starvation and virulence in mice but had enhanced pigment production and formation of quinolone tolerant persister cells. Our study is the first to report the complex relationship

TABLE 2 | List of genes differentially expressed in USA500 and Δ*ctaB* grown to 6 h.

Gene	Gene symbol	Log_2 fold change	*p*-value	Description
USA300HOU_RS*14135*		−2.04	4.4E-05	Hypothetical protein
USA300HOU_RS*09600*		−1.96	1.1E-03	Hypothetical protein
USA300HOU_RS*10850*		−1.83	1.2E-02	Hypothetical bacteriophage protein
USA300HOU_RS *10955*	*hld*	−1.53	8.5E-05	Delta-hemolysin
USA300HOU_RS *03725*		−1.42	9.0E-04	Hypothetical membrane protein
USA300HOU_RS *05170*		−1.24	1.9E-03	Hypothetical protein
USA300HOU_RS *04240*		−1.07	3.7E-02	Hypothetical protein
USA300HOU_RS *10770*		1.02	1.8E-02	Hypothetical protein
USA300HOU_RS *09400*	*ribD*	1.06	9.6E-07	Diaminohydroxyphosphoribosylaminopyrimidine deaminase
USA300HOU_RS *12385*	*ureB*	1.09	1.3E-02	Urease beta subunit
USA300HOU_RS *09390*	*ribA*	1.13	2.0E-07	Bifunctional 3,4-dihydroxy-2-butanone-4-phosphate synthase/GTP cyclohydrolase II
USA300HOU_RS *10800*		1.17	2.8E-03	Hypothetical bacteriophage protein
USA300HOU_RS *09395*	*ribB*	1.32	3.5E-08	Riboflavin synthase alpha subunit
USA300HOU_RS *13155*		1.32	1.6E-02	ABC superfamily ATP binding cassette transporter, ABC/membrane protein
USA300HOU_RS *06710*		1.32	2.3E-02	ABC superfamily ATP binding cassette transporter, membrane protein
USA300HOU_RS *03970*		1.39	4.5E-07	Iron (Fe+3) ABC superfamily ATP binding cassette transporter, membrane protein
USA300HOU_RS *12770*	*htrB*	4.14	1.2E-51	Hemin ABC superfamily ATP binding cassette transporter, ABC protein
USA300HOU_RS *12775*	*htrA*	4.34	1.6E-55	Hemin ABC superfamily ATP binding cassette transporter, membrane protein

between heme production, persister formation, and virulence in *S. aureus*.

We have shown that CtaB depletion barely affected growth in rich medium (TSB), but caused faster death under starvation stress (**Figure 3A**). The result echoes the finding by Clements et al., as CtaA mutation caused growth defect in glucose-limiting chemically defined medium (Clements et al., 1999). Heme production is a key step for cellular aerobic respiration and energy conversion, providing resources for synthesis of heme A-containing terminal oxidases (Svensson and Hederstedt, 1994; Hederstedt et al., 2005). The changes in the respiratory chain by mutation of CtaB could account for the defects. Meanwhile, it is more difficult to explain the enhanced pigment production caused by CtaB depletion. The production of staphyloxanthin, the main pigment of Staphylococci, is mediated by factors encoded by *crtOPQMN*, using FPP as the substrate (Wieland et al., 1994; Pelz et al., 2005). Regulators such as *rsbUVW-sigB* are known to regulate expression of pigment genes in *S. aureus* (Kullik et al., 1998; Giachino et al., 2001). In previous reports, suppression of genes from metabolic pathways (purine biosynthesis, the TCA cycle and oxidative phosphorylation) has also been found to affect pigment production (Lan et al., 2010). We detected the expression of pigment associated genes and found that CtaB deletion did not affect expression of *rsbUVW-sigB, fliA (sigB)* or *crtOPQMN*, while expression of *citZ* in *ΔctaB* mutant was down regulated (0.45) while *qoxB* was induced (2.09), and the other metabolic genes were not affected (**Table 4**). FPP is a key intermediate in mevalonate pathway that serves as a substrate of several pathways including synthesis of heme A and staphyloxanthin (Szkopinska and Plochocka, 2005). Since CtaB deletion did not affect pigment production by altering expression of the currently known genes of pigment production pathway, the possibility is worth considering that the absence of competition by heme A production pathway leaves more FPP to staphyloxanthin synthesis pathway, thus enhancing pigment production provided.

From the RNA-seq data, we show the down regulation of multiple virulence genes was caused by CtaB depletion. Despite the depression of global regulatory RNA RNAIII and several classic virulence factors (EsaB, EsaC, EsXB, etc.), DltA-D and most of the amino acid ABC transporters were down regulated. The four proteins (DltA-D) incorporate D-alanine into cell wall polymers during teichoic acid synthesis (Reichmann et al., 2013) and their inactivation has been shown to impact the defense of *S. aureus* against antimicrobial agents (Peschel et al., 1999). Expression of many amino acid transporter genes (*oppA1*, *oppC1*, *oppD1,* and *oppF1*) were found down regulated in the CtaB knockout strain. These ABC transporters not only function by obtaining nutrients, but play important roles in adherence and processing of secreting toxins (Podbielski et al., 1996). They also showed up frequently in screening of virulence genes of *S. aureus* with transposon libraries in different animal models (Mei et al., 1997; Coulter et al., 1998; Bae et al., 2004).

Pigment production has been found to enhance fitness and virulence and help the bacteria cope with oxidative stress (Clauditz et al., 2006). However, our results seem to contradict this finding of association of pigment production and virulence as we see enhanced pigment production of CtaB mutant but less virulence. Nevertheless, CtaB deletion had multiple effects on *S. aureus,* despite enhanced pigment production, it caused attenuated hemolytic activity and survival in animal model. It is likely the virulence attenuation of CtaB mutant is combined effect of more important attenuated hemolytic activity over the increased pigment production such that the net outcome is

TABLE 3 | List of genes differentially expressed in USA500 and ΔctaB grown to 24 h.

Gene	Gene symbol	log_2 fold change	*p*-value	Description
USA300HOU_RS01190		−3.37	3.36E-06	Acetyl-CoA C-acetyltransferase
USA300HOU_RS01195		−3.30	7.44E-06	3-hydroxyacyl-CoA dehydrogenase
USA300HOU_RS01200		−3.19	9.02E-06	Acyl-CoA dehydrogenase
USA300HOU_RS01205		−3.16	1.08E-05	Long-chain-fatty-acid–CoA ligase
USA300HOU_RS02265	*cobW1*	−2.78	6.77E-04	Cobalamin (vitamin B12) biosynthesis protein
USA300HOU_RS01210		−2.76	1.58E-04	3-oxoacid CoA-transferase
USA300HOU_RS11065	*ilvB1*	−2.69	1.10E-03	Acetolactate synthase large subunit
USA300HOU_RS02055	*xprT*	−2.66	1.47E-03	Xanthine phosphoribosyltransferase
USA300HOU_RS12265		−2.57	2.28E-03	Hypothetical protein
USA300HOU_RS14500	*lip*	−2.16	2.42E-03	Triacylglycerol lipase
USA300HOU_RS02060	*pbuX*	−2.16	3.25E-03	NCS2 family nucleobase:cation symporter-2
USA300HOU_RS05895		−2.14	1.26E-02	Antibacterial protein
USA300HOU_RS04630	*dltC*	−2.11	7.32E-03	D-alanine–poly(phosphoribitol) ligase
USA300HOU_RS08560		−2.07	1.27E-02	Acetyl-CoA carboxylase biotin carboxyl carrier subunit
USA300HOU_RS12135	*rpmC*	−2.04	3.26E-03	Ribosomal protein L29
USA300HOU_RS11075	*ilvC*	−2.03	1.12E-02	Ketol-acid reductoisomerase
USA300HOU_RS00655		−2.03	3.65E-02	Hypothetical membrane protein
USA300HOU_RS01875		−2.01	4.74E-02	Hypothetical protein
USA300HOU_RS04875	*fabH1*	−1.95	4.11E-03	3-oxoacyl-[acyl-carrier-protein] synthase
USA300HOU_RS02840	*rplL1*	−1.94	5.40E-03	Ribosomal protein L7/L12
USA300HOU_RS12130	*rpsQ*	−1.93	5.02E-03	Ribosomal protein S17
USA300HOU_RS04900	*oppD1*	−1.91	4.92E-03	Oligopeptide ABC superfamily ATP binding cassette transporter, ABC protein
USA300HOU_RS11060	*ilvD*	−1.89	1.79E-02	Dihydroxy-acid dehydratase
USA300HOU_RS07110	*dapB*	−1.84	1.91E-02	Dihydrodipicolinate reductase
USA300HOU_RS13735		−1.82	1.46E-02	Transcriptional regulator
USA300HOU_RS08760	*rplU*	−1.79	1.10E-02	Ribosomal protein L21
USA300HOU_RS07115	*dapD*	−1.75	2.17E-02	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase
USA300HOU_RS07100	*asd*	−1.74	2.52E-02	Aspartate-semialdehyde dehydrogenase
USA300HOU_RS06985	*trpB*	−1.72	4.42E-02	Tryptophan synthase beta subunit
USA300HOU_RS06055	*pyrE*	−1.71	2.74E-02	Orotate phosphoribosyltransferase
USA300HOU_RS06045	*carB*	−1.68	1.67E-02	Carbamoyl-phosphate synthase (glutamine-hydrolyzing), large subunit
USA300HOU_RS01940	*ssb1*	−1.67	1.64E-02	Single-stranded DNA-binding protein
USA300HOU_RS01120	*rplF*	−1.66	1.69E-02	Ribosomal protein L6
USA300HOU_RS02835	*rplJ*	−1.65	1.75E-02	Ribosomal protein L10
USA300HOU_RS02375	*gltB1*	−1.63	2.23E-02	Glutamate synthase (NADPH), large subunit
USA300HOU_RS06050	*pyrF*	−1.62	3.08E-02	Orotidine-5'-phosphate decarboxylase
USA300HOU_RS04905	*oppF1*	−1.58	1.92E-02	Oligopeptide ABC superfamily ATP binding cassette transporter, ABC protein
USA300HOU_RS01935	*rpsF*	−1.56	2.29E-02	Ribosomal protein S6
USA300HOU_RS04620	*dltA*	−1.55	2.45E-02	Long-chain-fatty-acid–CoA ligase
USA300HOU_RS07105	*dapA*	−1.52	4.99E-02	dihydrodipicolinate synthase
USA300HOU_RS04635	*dltD*	−1.51	3.00E-02	D-alanine transfer protein DltD
USA300HOU_RS07190		−1.51	4.86E-02	Nitric-oxide reductase
USA300HOU_RS12610	*hutI*	−1.50	2.99E-02	Imidazolonepropionase
USA300HOU_RS02380	*gltD*	−1.50	4.97E-02	Glutamate synthase (NADPH) small subunit
USA300HOU_RS05945	*murD*	−1.50	2.66E-02	UDP-N-acetylmuramoylalanine–D-glutamate ligase
USA300HOU_RS08555		−1.49	2.77E-02	Biotin carboxylase
USA300HOU_RS12615	*hutU*	−1.49	3.30E-02	Urocanate hydratase
USA300HOU_RS13410		−1.47	3.09E-02	Possible decarboxylase
USA300HOU_RS12085	*rpmD*	−1.46	2.95E-02	Ribosomal protein L30
USA300HOU_RS11770		−1.46	3.46E-02	Hypothetical membrane protein
USA300HOU_RS06545	*glpK*	−1.45	3.79E-02	Glycerol kinase

(Continued)

TABLE 3 | Continued

Gene	Gene symbol	log_2 fold change	*p*-value	Description
USA300HOU_RS03955	*nrdF*	−1.45	3.80E-02	Ribonucleoside-diphosphate reductase subunit beta
USA300HOU_RS12120	*rplX*	−1.41	4.01E-02	Ribosomal protein L24
USA300HOU_RS01785	*glpT*	−1.41	3.96E-02	MOP superfamily multidrug/oligosaccharidyl-lipid/polysaccharide flippase transporter
USA300HOU_RS11480	*pyrG*	−1.38	4.72E-02	CTP synthase
USA300HOU_RS06895	*parC*	−1.35	4.42E-02	DNA topoisomerase (ATP-hydrolyzing) ParC
USA300HOU_RS01150	*pfl*	1.33	4.65E-02	Formate C-acetyltransferase
USA300HOU_RS12815		1.37	4.46E-02	Hypothetical lipoprotein
USA300HOU_RS09730		1.39	4.50E-02	hypothetical bacteriophage protein
USA300HOU_RS14200	*nrdD*	1.42	3.47E-02	Anaerobic ribonucleotide reductase large subunit
USA300HOU_RS03820		1.46	3.56E-02	Hypothetical membrane protein
USA300HOU_RS05800		1.47	4.78E-02	Fibrinogen-binding protein
USA300HOU_RS03270		1.49	3.19E-02	Hydrolase
USA300HOU_RS12795		1.51	2.88E-02	Hypothetical membrane protein
USA300HOU_RS03030		1.51	2.58E-02	Hypothetical protein
USA300HOU_RS01345	*scdA*	1.52	2.94E-02	Cell division and morphogenesis protein ScdA
USA300HOU_RS11555		1.54	3.52E-02	Hypothetical protein
USA300HOU_RS12775	*htrA*	1.55	4.66E-02	Hemin ABC superfamily ATP binding cassette transporter, membrane protein
USA300HOU_RS12700		1.56	3.28E-02	Hypothetical protein
USA300HOU_RS03830		1.61	4.27E-02	Hypothetical membrane protein
USA300HOU_RS13375		1.62	2.50E-02	Oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
USA300HOU_RS13655		1.62	3.16E-02	Possible hydrolase
USA300HOU_RS07060	*pstA*	1.63	3.71E-02	Phosphate ABC superfamily ATP binding cassette transporter, membrane protein
USA300HOU_RS08965		1.66	1.63E-02	Sensor histidine kinase
USA300HOU_RS05785		1.69	4.13E-02	Hypothetical protein
USA300HOU_RS13875		1.71	1.13E-02	Hypothetical protein
USA300HOU_RS11550	*dps*	1.71	1.41E-02	Dps family stress protein
USA300HOU_RS14195	*nrdG*	1.72	1.43E-02	Anaerobic ribonucleotide reductase small subunit
USA300HOU_RS01630		1.72	3.52E-02	Acid phosphatase
USA300HOU_RS13840		1.77	2.44E-02	FeoB family ferrous iron (Fe2+) uptake protein
USA300HOU_RS13100	*hlgC*	1.80	1.30E-02	Gamma hemolysin component C
USA300HOU_RS06060		1.83	4.16E-02	Hypothetical protein
USA300HOU_RS01155	*pflA*	1.85	6.42E-03	[Formate-C-acetyltransferase]-activating enzyme
USA300HOU_RS12770	*htrB*	1.86	2.23E-02	Hemin ABC superfamily ATP binding cassette transporter, ABC protein
USA300HOU_RS14125	*fdaB*	1.87	6.17E-03	Fructose-bisphosphate aldolase
USA300HOU_RS13320		1.89	2.63E-02	Hypothetical protein
USA300HOU_RS01220		1.89	1.78E-02	ABC superfamily ATP binding cassette transporter, binding protein
USA300HOU_RS02105	*set3*	1.91	1.38E-02	Staphylococcal exotoxin
USA300HOU_RS01390		1.93	2.02E-02	Hypothetical protein
USA300HOU_RS13085		1.96	4.09E-03	Immunoglobulin G-binding protein SBI
USA300HOU_RS00580	*spa*	1.99	3.51E-03	Immunoglobulin G binding protein A
USA300HOU_RS00555		2.09	3.39E-03	Myosin-cross-reactive antigen
USA300HOU_RS14135		2.14	3.07E-02	Hypothetical protein
USA300HOU_RS10525	*mapW2*	2.14	4.61E-03	Cell surface protein MapW2
USA300HOU_RS12790		2.18	5.84E-03	Hypothetical protein
USA300HOU_RS07065	*pstC*	2.23	4.71E-03	Phosphate ABC superfamily ATP binding cassette transporter, membrane protein
USA300HOU_RS01225		2.26	9.38E-03	Hypothetical protein
USA300HOU_RS01665		2.29	2.74E-03	Possible CNT family concentrative nucleoside transporter
USA300HOU_RS13665		2.29	3.12E-03	MarR family transcriptional regulator
USA300HOU_RS13095	*hlgA*	2.30	8.97E-04	Gamma-hemolysin component A
USA300HOU_RS01655		2.35	2.51E-03	PfkB family carbohydrate kinase

(Continued)

TABLE 3 | Continued

Gene	Gene symbol	log_2 fold change	*p*-value	Description
USA300HOU_RS03725		2.41	1.29E-02	Hypothetical membrane protein
USA300HOU_RS04290	*nuc*	2.48	3.60E-03	Micrococcal nuclease
USA300HOU_RS13635		2.50	2.35E-03	ABC superfamily ATP binding cassette transporter, membrane protein
USA300HOU_RS13830	*clp*	2.51	2.93E-04	S14 family endopeptidase Clp
USA300HOU_RS13660		2.53	1.74E-03	Possible lactoylglutatione lyase
USA300HOU_RS01660		2.62	9.10E-04	Hypothetical protein
USA300HOU_RS05775		2.62	2.08E-04	Hypothetical protein
USA300HOU_RS05855		2.80	1.39E-02	Exotoxin
USA300HOU_RS10450		2.80	8.14E-04	Hypothetical protein
USA300HOU_RS07070	*pstS*	2.85	5.80E-05	Phosphate ABC superfamily ATP binding cassette transporter, binding protein
USA300HOU_RS05795		2.90	8.96E-05	Fibrinogen-binding protein
USA300HOU_RS01235	*hmp*	2.96	5.40E-05	Possible nitric oxide dioxygenase
USA300HOU_RS10965	*agrD*	3.45	6.02E-04	Accessory gene regulator protein D
USA300HOU_RS04615		4.03	5.89E-03	Hypothetical protein
USA300HOU_RS13630		4.07	6.62E-06	ABC superfamily ATP binding cassette transporter, ABC protein
USA300HOU_RS01365	*lrgB*	4.08	9.13E-08	Murein hydrolase regulator LrgB
USA300HOU_RS01360	*lrgA*	4.36	1.25E-08	Murein hydrolase regulator LrgA
USA300HOU_RS01230		6.07	1.16E-06	Hypothetical protein

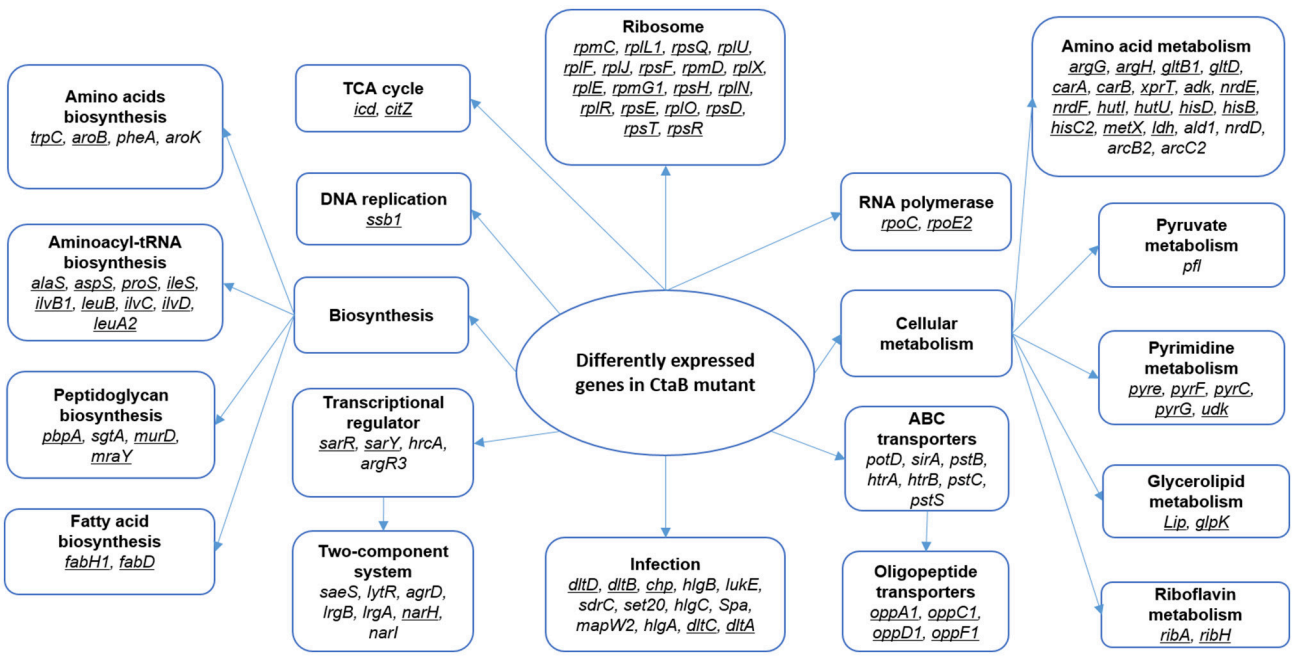

FIGURE 6 | Proposed pathways affected by depletion of CtaB in stationary phase *S. aureus*. Pathways enriched in genes with higher than 2-fold change in CtaB mutant at 24 h are framed and bold. Genes that were down regulated in the CtaB mutant are underlined, while those up regulated not underlined.

still attenuated virulence despite increased pigment production which is often associated with virulence.

Persister formation is a phenomenon with highly complex mechanisms. Energy production and protein translation are two vital pathways for bacterial survival and reproduction, and it is generally believed that an overall suppression of metabolism and replication is a universal cause for bacterial persister formation (Lewis, 2012; Kwan et al., 2013). In *E. coli*, deficiency of energy production genes such as *sucB* and *ubiF* has been found to decrease persister survival (Ma et al., 2010). It has also been shown in *E. coli* that bacteriostatic antibiotic treatment enhances persister formation via suppression of

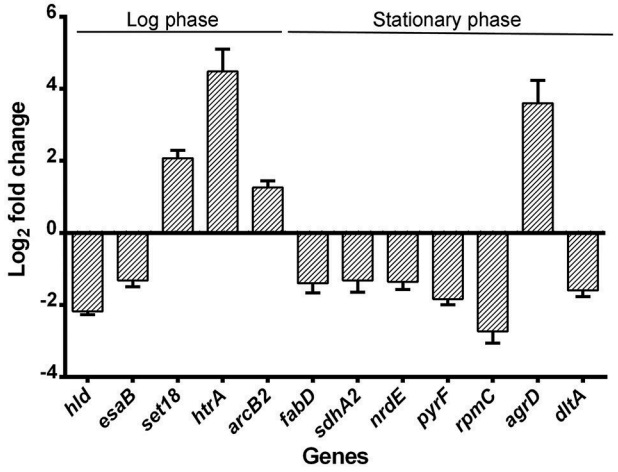

FIGURE 7 | Validation of RNA-seq by quantitative real-time PCR. Relative mRNA levels of transcripts corresponding to USA500 and Δctab mutant grown to 6 or 24 h were determined. RNA was obtained from the same samples for RNA-seq and experiments were performed in triplicate. Bars show the fold change of *ΔctaB* mutant vs. USA500 and error bars indicate standard deviations calculated with the $2^{-\Delta\Delta CT}$ method based on three independent experiments.

TABLE 4 | Transcription change of pigment production associated genes.

Genes	Fold change (*ΔctaB* vs. USA500)	*SD*
rsbU	0.90	0.122
crtM	1.18	0.143
qoxB	2.09	0.276
citZ	0.45	0.089
fliA (sigB)	0.91	0.129
purA	0.56	0.113
USA300HOU_0726 (NWMN_0672)	1.38	0.127
USA300HOU_ (NWMN_1144)	3.54	0.323

cellular respiration (Lobritz et al., 2015). In *S. aureus*, a recent study correlated the drop of ATP level to enhanced persister formation in stationary phase (Pontes et al., 2015). CtaB is a key factor in *S. aureus* respiratory chain and energy production. In stationary phase when most glucose is consumed, *S. aureus* turns to utilize amino acids such as arginine and histidine for energy production (Makhlin et al., 2007). We also found that in stationary phase, multiple genes involved in amino acid metabolism (*argG*, *hutI*, *hisD*, etc.) were inhibited in *ΔctaB* strain (**Table 3**). Based on these findings, CtaB depletion might account for augumented persister formation. However, the correlation between respiration and persister formation is far from unveiled. A counter-example has been provided by Mehmet et al. who reported that inhibition of respiration during stationary phase with KCN reduced persister levels in *E. coli* (Orman and Brynildsen, 2015).

While more work needs to be done to investigate the role of respiratory chain in persister formation, it is also important to further investigate how repression of protein production affects persister formation. The well-understood mechanism of HipAB Toxin-antitoxin system affecting persister formation in *E. coli*, relies on (p)ppGpp to trigger a regulatory cascade involving inorganic polyphosphate (polyP) and Lon, which eventually results in accumulation of (p)ppGpp and persister formation (Rodionov and Ishiguro, 1995; Korch et al., 2003; Germain et al., 2013; Maisonneuve et al., 2013). In our study, CtaB depletion caused strong inhibition of translation by repressing genes involved in multiple aspects of protein production, including amino acid transport (*oppA1*, *oppC1*, *oppD1*, etc.), amino acid synthesis (trpC, aroB,), aminoacyl-tRNA biosynthesis (*aspS*, *alaS*, *ileS*, etc.) and ribosome proteins (*rpmC*, *rplF*, *rpsE*, etc.) (**Table 3**; **Figure 6**).

It is generally assumed that elevated persistence is associated with better survival and therefore higher virulence in animal models. However, our observation that mutation of CtaB caused attenuated virulence but elevated persister formation, seems paradoxical. Indeed, many infectious diseases are difficult to be treated with antibiotics due to persisters but not resistance (Mulcahy et al., 2010; Welsh et al., 2011). However, we propose that in most cases with MRSA infection the role of virulence is greater than persister formation because: the proportion of persisters is generally small; after antibiotics kill the majority of infecting population of bacteria, the host immune system generally eliminates persisters non-selectively. Nevertheless, the importance of persister formation by MRSA should not be neglected. Future studies are needed to better understand the roles virulence and persistence play in *S. aureus* infection.

In our study, the CtaB mutant only showed elevated persister formation with levofloxacin and ciprofloxacin but no other antibiotics. This may be attributed to the multi-drug resistance of the MRSA strain, which may have masked the defect in persistence to other antibiotics. The difference in persister formation between MRSA and methicillin-sensitive *S. aureus* (MSSA) is worth further investigation.

Our study suggests the importance of heme synthesis in virulence and persister formation of *S. aureus* and provide new insights into the role of CtaB in bacterial respiration in *S. aureus* virulence and persistence. However, one limitation of the study is that we have not dealt specifically with the metabolic aspects of CtaB mutation, such as the efficiency of the respiratory chain in the mutant and possible changes in components of the TCA cycle as well as comparing the phenotypes of *S. aureus* and the *ctaB* mutant in anaerobic conditions. Future studies are needed to address these issues and better understand the relationship between *S. aureus* respiration and virulence and persistence.

AUTHOR CONTRIBUTIONS

YZ, TX, and WZ designed the work and revised the manuscript; TX, JH, JZ, JC, and NW completed all the experiments; TX and JH performed the statistically analysis and made the figures; TX and YZ wrote the manuscript.

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Polyphosphate Kinase Mediates Antibiotic Tolerance in Extraintestinal Pathogenic *Escherichia coli* PCN033

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Extraintestinal pathogenic *Escherichia coli* (ExPEC) causes a variety of acute infections in its hosts, and multidrug-resistant strains present significant challenges to public health and animal husbandry. Therefore, it is necessary to explore new drug targets to control *E. coli* epidemics. Previous studies have reported that *ppk* mutants of *Burkholderia pseudomallei* and *Mycobacterium tuberculosis* are more susceptible than the wild types (WTs) to stress. Therefore, we investigated the stress response to antibiotics mediated by polyphosphate kinase (PPK) in ExPEC strain PCN033. We observed that planktonic cells of a *ppk* knockout strain (Δ*ppk*) were more susceptible to antibiotics than was WT. However, biofilm-grown Δ*ppk* cells showed similar susceptibility to that of the WT and were more tolerant than the planktonic cells. During the planktonic lifestyle, the expression of genes involved in antibiotic tolerance (including resistance-conferring genes, and antibiotic influx, and efflux genes) did not change in the Δ*ppk* mutant without antibiotic treatment. However, the resistance-conferring gene *bla* and efflux genes were upregulated more in the WT than in the Δ*ppk* mutant by treatment with tazobactam. After treatment with gentamycin, the efflux genes and influx genes were upregulated and downregulated, respectively, more in the WT than in the Δ*ppk* mutant. The expression of genes involved in biofilm regulation also changed after treatment with tazobactam or gentamycin, and which is consistent with the results of the biofilm formation. Together, these observations indicate that PPK is important for the antibiotic stress response during the planktonic growth of ExPEC and might be a potential drug target in bacteria.

Keywords: ExPEC, antibiotic resistance, PPK, gene expression, RNA-seq, qPCR

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INTRODUCTION

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is a major cause of urinary tract infections in women, abdominal sepsis, and septicemia in elderly or immunocompromised individuals, and meningitis in newborns, with high morbidity and mortality (Gaschignard et al., 2011; Weston et al., 2011; Mellata, 2013). ExPEC strains commonly colonize domestic animals, such as pigs, chickens, and cattle, causing significant losses in animal husbandry and threating human health (Girardeau et al., 2003; Johnson et al., 2005; Bergeron et al., 2012). We previously investigated the prevalence

of ExPEC in swine across China and detected ExPEC in 10.1% of porcine samples. The frequency of ExPEC isolated from pigs increased between 2004 and 2007 from 3.1 to 14.6% (Tan et al., 2012). The emergence of multidrug-resistant strains has significantly hindered the prevention and control of ExPEC epidemics (Sedláková et al., 2014; Sidrach-Cardona et al., 2014). Therefore, it is urgent that we identify new drug targets to control these increasing *E. coli* outbreaks.

Polyphosphate kinase (PPK) is an essential enzyme in polyphosphate (polyP) synthesis and has been implicated in many intracellular biological processes. *Pseudomonas aeruginosa* in which *ppk* was deleted showed impairments in motility, quorum sensing, and virulence (Rashid et al., 2000), compacted nucleoids, membrane distortion, extracellular polymer production, and a susceptibility to desiccation (Fraley et al., 2007). PPK also plays a prominent role in the stress response, and a *Burkholderia pseudomallei ppk* mutant was susceptible to hydrogen peroxide under oxidative stress conditions (Tunpiboonsak et al., 2010). A *ppk1* mutant strain of *Mycobacterium tuberculosis* displayed a survival defect in response to nitrosative stress, and the negligible levels of polyP were associated with its increased susceptibility to certain tuberculosis drugs (Singh et al., 2013). PPK is highly conserved in bacteria, but is absent in higher mammals (Brown and Kornberg, 2004), indicating that PPK has potential utility as an antibacterial drug target.

As an opportunistic pathogen, *E. coli* mainly causes acute infections in immunocompromised individuals (Chaudhuri and Henderson, 2012; Mellata, 2013); further, acute infections are associated with its planktonic growth mode (Li et al., 2014). Therefore, we explored the role of PPK in antibiotic resistance in the planktonic cells of ExPEC strain PCN033. Biofilm formation contributes to chronic bacterial infections, such as the recurrent pyelonephritis caused by uropathogenic *E. coli* in children (Tapiainen et al., 2014). Therefore, we also studied the role of PPK in antibiotic tolerance in biofilm-grown cells.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The wild-type (WT) strain used in this study, PCN033, was isolated from a diseased swine in Hubei Province, Central China (Liu et al., 2015). The Δ*ppk* mutant was obtained by in-frame deletion with the suicide plasmid pRE112 (He et al., 2012). Details of both these strains and the primers used in this study are listed in **Table S1** (available as Supplementary Data). The antibiotic susceptibility and biofilm formation assays were performed in MOPS broth (an inorganic phosphorus [P_i]-limited medium) at 28°C (Neidhardt et al., 1974). The antibiotics used in the biofilm inhibition assay were added below the minimum inhibitory concentrations (MICs), and had no bactericidal effect on the planktonic cells.

Growth Characteristics

Fresh colonies of both strains were taken from Luria–Bertani (LB) agar plates, used to inoculate LB broth, and then cultured in MOPS in a shaker incubator for 12 h. The growth characteristics were monitored turbidimetrically at 600 nm on a spectrophotometer (Eppendorf, Hamburg, Germany) and the colony-forming units (cfu) were counted at 1 h intervals. The generation times were calculated with the formula (Penfold and Norris, 1912):

$$G = \frac{T}{Log_2^{\frac{b}{a}}}$$

where G is the generation time; T is the length of the logarithmic phase; a is the initial number of bacteria; and b is the final number of bacteria.

Susceptibility Assay

Each MIC was determined with a series of two-fold dilutions of the antibiotic in MOPS broth, according to the Clinical Laboratory and Standards Institute guidelines. A pre-grown inoculum of each strain was diluted in MOPS to a final concentration of 10^7 cfu/mL, and the concentration of antibiotic added varied from 0.25 to 512 mg/L. The plates were incubated for 24 h, and the MICs were determined as the lowest antibiotic concentrations that produced no visible growth.

The susceptibility assay of the biofilms was performed as described previously (Benthall et al., 2015), with some modifications. The MIC on the biofilm was determined by allowing a biofilm to form in a 96-well-plate for 24 h. The unattached cells were washed off three times with 0.9% saline. The biofilm was incubated for 24 h with a range of antibiotic concentrations from 512 to 0.25 mg/L. The MIC was defined as the lowest antibiotic concentration at which no bacterial growth was detected. To determine the effect of the biofilm on the bacterial susceptibility to antibiotics, the viability of planktonic and biofilm-grown cells was calculated after the antibiotic treatments. About 10^7 cfu were incubated with antibiotic concentrations of 2 × MIC for 3 h, and the cfu were then counted.

To quantify the bactericidal activity of these antibiotics on the biofilm, their activity percentage was assessed according to a previous report (Sánchez-Gómez et al., 2015), with some modifications. The ability of these antibiotics to remove the biofilm attached to the microplate was determined with crystal violet (CV) staining. For this purpose, the treated biofilm was stained with CV for 30 min at room temperature. The excess stain was then rinsed off with saline and the CV remaining on the biofilm was dissolved in 33% acetic acid. The absorbance was measured at 595 nm with a Synergy HT microplate reader (BioTek, USA). The activity percentage was calculated according to Sánchez-Gómez et al., with the formula:

$$Activity\ Percentage = \frac{(C-B)-(T-B)}{C-B}\ \text{x}\ 100$$

where C is the absorbance of the control well-containing untreated biofilm; T is the absorbance of the well-containing treated biofilm; and B is the absorbance of the blank well (i.e., no biofilm).

Biofilm Formation Assay

Static cultures of biofilm grown at 28°C were analyzed in flat-bottom 96-well-microtiter plates (Corning, USA) using CV. Briefly, approximately 10^7 cfu were inoculated with sub-MIC antibiotics and cultured for 24 h. The unattached cells were then washed off as described above, fixed with absolute ethanol for 30 min, dried, and stained with 0.1% CV solution for 1 h. The microplates were then washed three times to remove any unattached CV. The CV in the stained biofilm was then dissolved in 33% acetic acid solution and the absorbance read at 595 nm. Each experiment was repeated twice with three technical replicates.

RNA-seq Assay and Quantitative Real-Time (qRT)–PCR Validation

RNA samples from each strain were prepared for RNA sequencing. Sequencing was performed on an Illumina Hiseq 2500 sequencer (Illumine Inc.) by Shanghai Hanyu Biotechnology Co., Ltd (Shanghai, China). The RNA-seq results were confirmed with qRT–PCR. Before qRT-PCR, the RNA of both strains was extracted with RNAiso Plus reagent (Takara, China). Any genomic DNA contamination was eliminated, and the RNA was reverse transcribed to cDNA with the PrimeScript^{TM}RT reagent Kit with gDNA Eraser (Takara). Quantitative real-time PCR was performed in triplicate in optical 96-well-reaction plates (Life Technologies, China) using Power SYBR Green PCR Master Mix (Life Technologies). The primers are listed in **Table S1**. The mRNA levels of the target genes were normalized to the internal 16S rRNA control with the ΔΔCt method (Kubista et al., 2006). The planktonic cells of the WT and Δ*ppk* mutant were cultured to an optical density at a wavelength of 600 nm (OD_{600}) of about 0.5. Tazobactam or gentamycin was added at a concentration of 2 × MIC and the cells were incubated for 2 h. The cells were then collected for RNA extraction and qRT–PCR.

Statistical Analysis

Statistical analysis was performed with the SPSS software (SPSS, Inc., Chicago, IL, USA) on a Windows XP system. Biofilm formation, differential expression, and generation times were compared with one-way analysis of variance (ANOVA). Values are expressed as means ± *SD*, and statistically significant differences are marked with asterisks. To identify the levels of gene expression, the RNA-seq data were analyzed with an MA-plot-based method with the random sampling model (MARS) in the DEGseq software (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html). Genes showing two-fold changes in expression, a false discovery rate <0.001, and reads per kilobase per million >20 in at least one sample were considered to be differentially expressed under the conditions used.

RESULTS

Growth Characteristics

In MOPS minimal medium, the density of the Δ*ppk* cultures was slightly higher than that of the WT at each time point tested (**Figure 1**). However, the generation times, calculated from both the optical density and cfu, for Δ*ppk* ($G_{OD} = 56.5 \pm 4.73$, $G_{CFU} = 42.0 \pm 1.88$) were not significantly different ($P > 0.05$) from those for WT ($G_{OD} = 56.3 \pm 3.64$, $G_{cfu} = 45.9 \pm 4.35$).

Planktonic Δ*ppk*Cells are More Susceptible to Antibiotics than WT Cells

As shown in **Table 1**, the 17 antibiotics screened in this study were categorized based on their targets: cell wall biosynthesis (type A), protein biosynthesis (type B), nucleotide metabolism (type C), and cell membrane (type D). There were no significant differences in the MIC values for the Δ*ppk* strain and WT in LB broth. However, in MOPS broth, Δ*ppk* was more susceptible to antibiotics than WT. Specifically, Δ*ppk* was much more susceptible to type B antibiotics than was WT, followed by type A antibiotics. Because the WT strain can accumulate more polyP in MOPS broth than in other medi (Ault-Riché et al., 1998), we performed all further assays in MOPS.

Biofilm-Grown Δ*ppk* and WT Cells Are Similarly Tolerant and More Tolerant than Planktonic Cells

To clarify the role of biofilms in antibiotic resistance, an MIC assay using biofilm-grown cells was performed as described previously (Benthall et al., 2015). As shown in **Table 2**, biofilm-grown Δ*ppk* cells showed almost no difference from WT cells in their antibiotic susceptibility, and biofilm-grown cells of both strains were more tolerant than the corresponding planktonic cells. Consistent with this, there was no significant difference in the ability of antimicrobial compounds to kill biofilm-grown cells of the WT and Δ*ppk* strains (**Figure S1**). However, more planktonic cells of the Δ*ppk* strain were killed than WT cells (**Figure 2**). In a future study, we will investigate the role of PPK in antibiotic tolerance within the planktonic growth mode.

Expression of Antibiotic-Resistance Genes without Antibiotic Treatment

RNA-seq data for the Δ*ppk* and WT strains regarding the expression of genes involved in antibiotic resistance, including resistance-conferring genes and antibiotic efflux and influx genes, are presented in **Table S2**. Of 53 genes known to be involved in antibiotic resistance or multidrug resistance, the expression of one resistance-conferring gene (*tetB*), two efflux genes (*mdtE* and *mdtG*), and one influx gene (*ompC*) was upregulated, and the expression of three efflux genes (*marA*, *marB*, and *mdtA*) was downregulated in the mutant compared with their expression in WT (**Figure 3A**). The expression of some of these genes was confirmed with qRT–PCR (**Figure 3B**).

Expression of Resistance-Conferring Genes after Antibiotic Treatment

Five resistance-conferring genes were selected for analysis when WT was treated with tazobactam, as shown in **Figure 4**. The expression of beta-lactamase (*bla*) was upregulated, as was that of aminoglycoside 3′N-acetyltransferase III (*aac*), fused UDP-L-Ara4N formyltransferase (*arnA*), and nitroreductase A

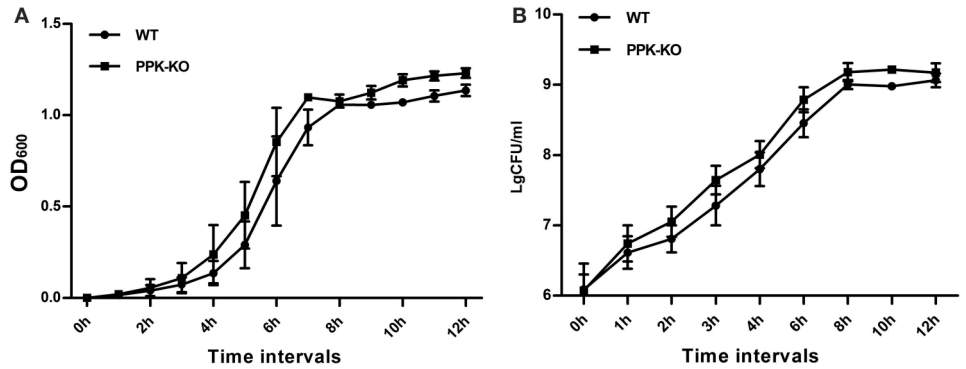

FIGURE 1 | Growth characteristics of *E. coli* strains PCN033 (wild type) and Δ*ppk* in MOPS broth: (A) optical density, and (B) colony-forming units.

TABLE 1 | Assay of the susceptibility of planktonic cells to antibiotics (mg/L).

Target (type)	antibiotic category	compound	MIC in LB		MIC change (fold)	MIC in MOPS		MIC fold (fold)	References
			PCN033	Δ*ppk*		PCN033	Δ*ppk*		
Cell wall biosynthesis(A)	β-lactams	cefotaxime	2	1	2	16	4	4	Bush, 2012
		ceftazidime	256	256	1	512	512	1	
		ampicillin	>512	>512	1	>512	>512	1	
		cefazolin	>512	>512	1	>512	>512	1	
		tazobactam	>512	>512	1	>512	256	>2	
		ticarcillin	>512	>512	1	>512	>512	1	
	Glycopeptide	vancomycin	>512	>512	1	128	64	2	
Protein biosynthesis (B)	Aminoglycosides	gentamicin	>512	>512	1	256	32	8	Davis, 1987
		gentamicin sulfate	>512	>512	1	256	64	4	
		amikacin	>512	>512	1	>512	64	≥4	
	Macrolide	Erythromycin	512	512	1	512	128	4	Brisson-Noël et al., 1988
Nucleotide metabolism (C)	Quinolones	norfloxacin	>512	512	>1	512	512	1	Aldred et al., 2014
		levofloxacin	128	64	2	64	16	4	
	Sulfonamides	Trimethoprim	128	128	1	256	128	2	Pérez-Trallero and Iglesias, 2003
		sulfadiazine	>512	>512	>1	>512	>512	1	
	Nitrofurans	macrodantin	512	256	2	128	128	1	Hof, 1988
Membrane (D)	Lipopeptde	polymyxin B	0.5	≤0.25	≥2	2	1	2	Grau-Campistany et al., 2015

(*nfsA*). The *bla*, *arnA*, and sulfate adenylyltransferase (*cysN*) genes were also upregulated in the Δ*ppk* mutant after tazobactam treatment, but their expression was higher in WT than in the Δ*ppk* mutant. The expression of resistance-conferring gene was not significantly altered by gentamycin treatment, except for *cysN*.

Expression of Genes Involved in Antibiotic Transport Was Altered More Strongly in WT than in the Δ*ppk* Mutant

The expression of six genes involved in antibiotic efflux was determined, as shown in **Figure 5A**. The efflux genes were upregulated in both the WT and Δ*ppk* mutant strains by tazobactam and gentamycin. However, the expression of *acrA*, *cusC*, and *marA* was higher in WT than in the Δ*ppk* mutant after treatment with tazobactam. After treatment with gentamycin, the expression of *acrA* and *marA* was also higher in WT than in the Δ*ppk* mutant. The expression of three genes involved in antibiotic influx was determined, as shown in **Figure 5B**. All the genes tested were downregulated by antibiotic treatment. However, *ompF* expression decreased more strongly in WT than in the Δ*ppk* mutant when treated with tazobactam. The expression of *ompF*, *ompC*, and *phoE* also decreased more strongly in WT than in the Δ*ppk* mutant after treatment with gentamycin.

Effects of Tazobactam and Gentamycin in Biofilm Formation

The RNA-seq data showed that the transcription levels of some genes associated with biofilm formation were altered

TABLE 2 | Assay of the susceptibility of biofilm-grown cells to antibiotics (mg/L).

Target (type)	antibiotic category	compound	MIC for biofilm		MIC change (fold)
			PCN033	Δ*ppk*	
Cell wall biosynthesis (A)	β-lactams	cefotaxime	128	256	0.5
		ceftazidime	512	512	1
		ampicillin	>512	>512	1
		cefazolin	>512	>512	1
		tazobactam	512	>512	≤0.5
		ticarcillin	>512	>512	1
Protein biosynthesis (B)	Aminoglycosides	gentamicin	512	512	1
		gentamicin sulfate	512	256	2
		amikacin	512	512	1
	Macrolide	erythromycin	512	512	1
	Quinolones	norfloxacin	512	512	1
		levofloxacin	32	32	1
Nucleotide metabolism (C)	Sulfonamides	Trimethoprim	512	512	1
		sulfadiazine	>512	>512	1
	Nitrofurans	macrodantin	512	512	1
Membrane (D)	Lipopeptde	polymyxin B	4	4	1

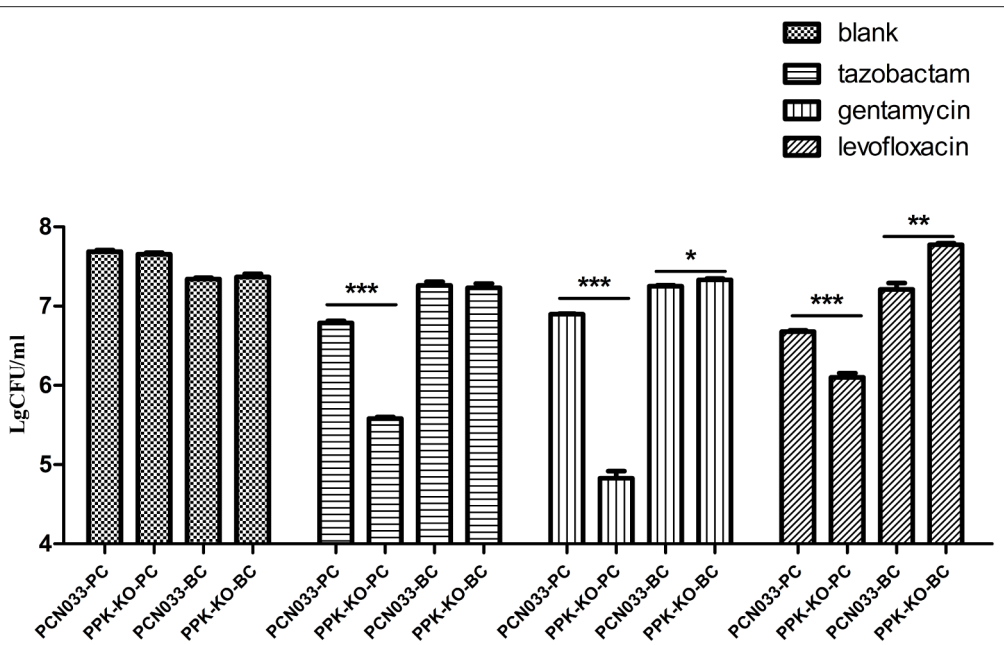

FIGURE 2 | Antibiotic killing assay of planktonic and biofilm-grown cells. PCN033-PC indicates planktonic WT cells; PPK-KO-PC indicates planktonic cells of the Δ*ppk* mutant; PCN033-BC indicates biofilm-grown WT cells; PPK-KO-BC indicates biofilm-grown cells of the Δ*ppk* mutant; blank indicates no antibiotic treatment. ***$p < 0.000$, **$p < 0.01$, *$p < 0.05$.

in the Δ*ppk* strain (**Tables S3**, **S4**), especially those encoding the flagella cluster, which simultaneously promotes biofilm generation and impedes biofilm maturation (Laverty et al., 2014). The expression of fimbrial and curli genes was also reduced in the Δ*ppk* mutant. The expression levels of some genes were confirmed with qRT–PCR (**Figure 6A**). The expression of four genes (*yddV*, *mcbR*, *bolA*, and *csgD*) involved in biofilm regulation was determined during antibiotic treatment, as shown in **Figure 6B** (Laverty et al., 2014; Lord et al., 2014; Dressaire et al., 2015; Wu et al., 2015). The expression of *yddV*, *mcbR*, and *bolA* was upregulated in WT but downregulated in the Δ*ppk* mutant when treated with tazobactam, and all four genes were upregulated in WT but downregulated in the Δ*ppk* mutant when treated with gentamycin. Biofilm formation was also evaluated in the presence of tazobactam or gentamycin, as shown **Figure 6C**. Biofilm formation increased in WT planktonic

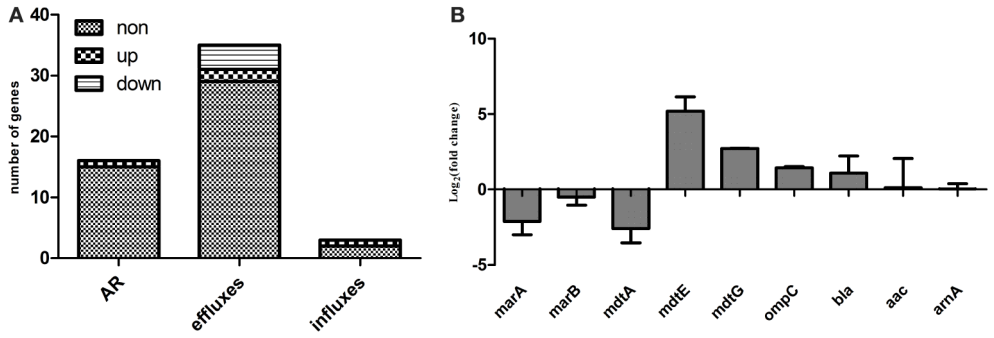

FIGURE 3 | Expression levels of genes involved in antibiotic resistance, antibiotic efflux, or antibiotic influx. (A) Expression levels determined with RNA-seq; **(B)** qRT–PCR confirmation. AR indicates genes that confer resistance, including beta-lactamase *bla*, aminoglycoside 3′N-acetyltransferase III (*aac*) etc.; efflux genes include *acrAB–tolC*, *acrDEF*, *cusCFBA*, *emrAB*, *emrKY*, *mdtABCEF*, etc.; and influx genes include *ompC*, *ompF*, and *phoE,* details are available in **Table S2**.

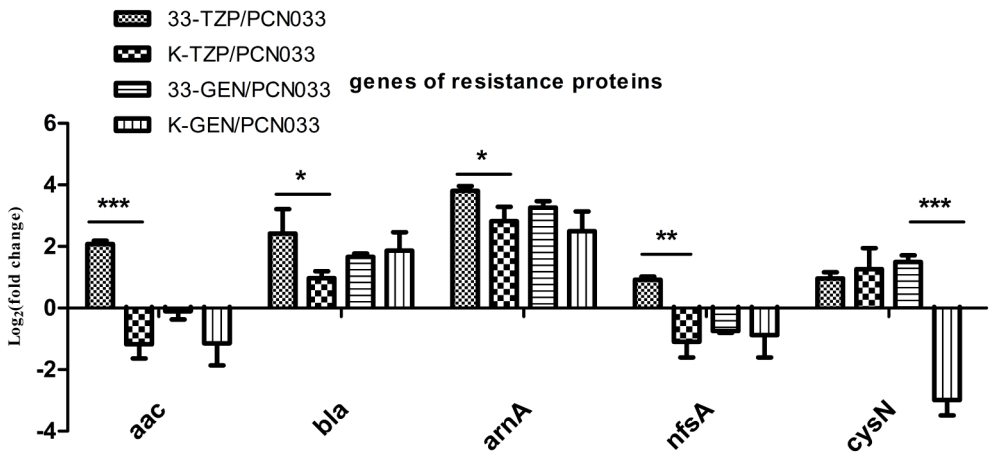

FIGURE 4 | Expression levels of genes that confer resistance, during tazobactam and gentamycin treatment. Expression levels were compared with those of WT (PCN033). 33-TZP/PCN033 indicates the expression levels in PCN033 during tazobactam treatment compared with those in untreated PCN033; K-TZP/PCN033 indicates expression levels in the Δ*ppk* mutant during tazobactam treatment compared with those in untreated PCN033; 33-GEN/PCN033 indicates expression levels in PCN033 during gentamycin treatment compared with those in untreated PCN033; K-GEN/PCN033 indicates expression levels in the Δ*ppk* mutant during gentamycin treatment compared with those in untreated PCN033. ***$p < 0.000$, **$p < 0.01$, *$p < 0.05$.

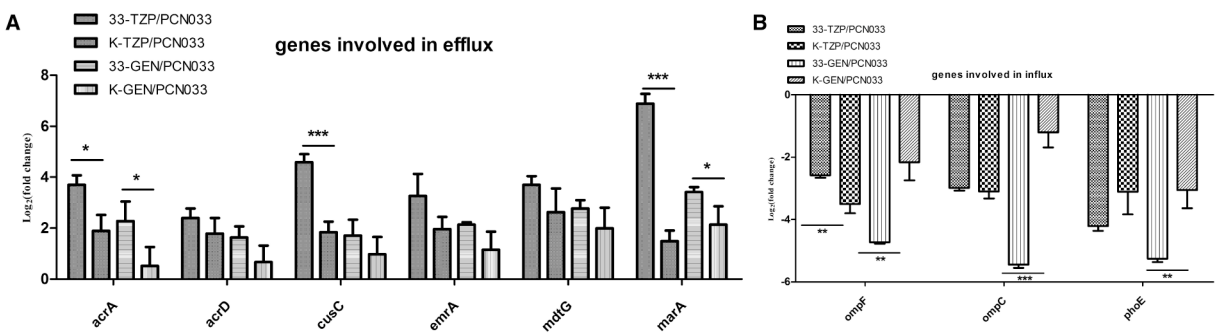

FIGURE 5 | Expression levels of genes involved in antibiotic efflux and influx during tazobactam and gentamycin treatments. (A) Expression of efflux genes, **(B)** expression of influx genes. Expression levels of the genes were compared with those of WT (PCN033). 33-TZP/PCN033 indicates the expression levels in PCN033 during tazobactam treatment compared with those in untreated PCN033; K-TZP/PCN033 indicates the expression levels in the Δ*ppk* mutant during untreated PCN033.K-GEN/PCN033 indicates the expression levels in the Δ*ppk* mutant during gentamycin treatment compared with those in untreated PCN033. ***$p < 0.000$, **$p < 0.01$, *$p < 0.05$.

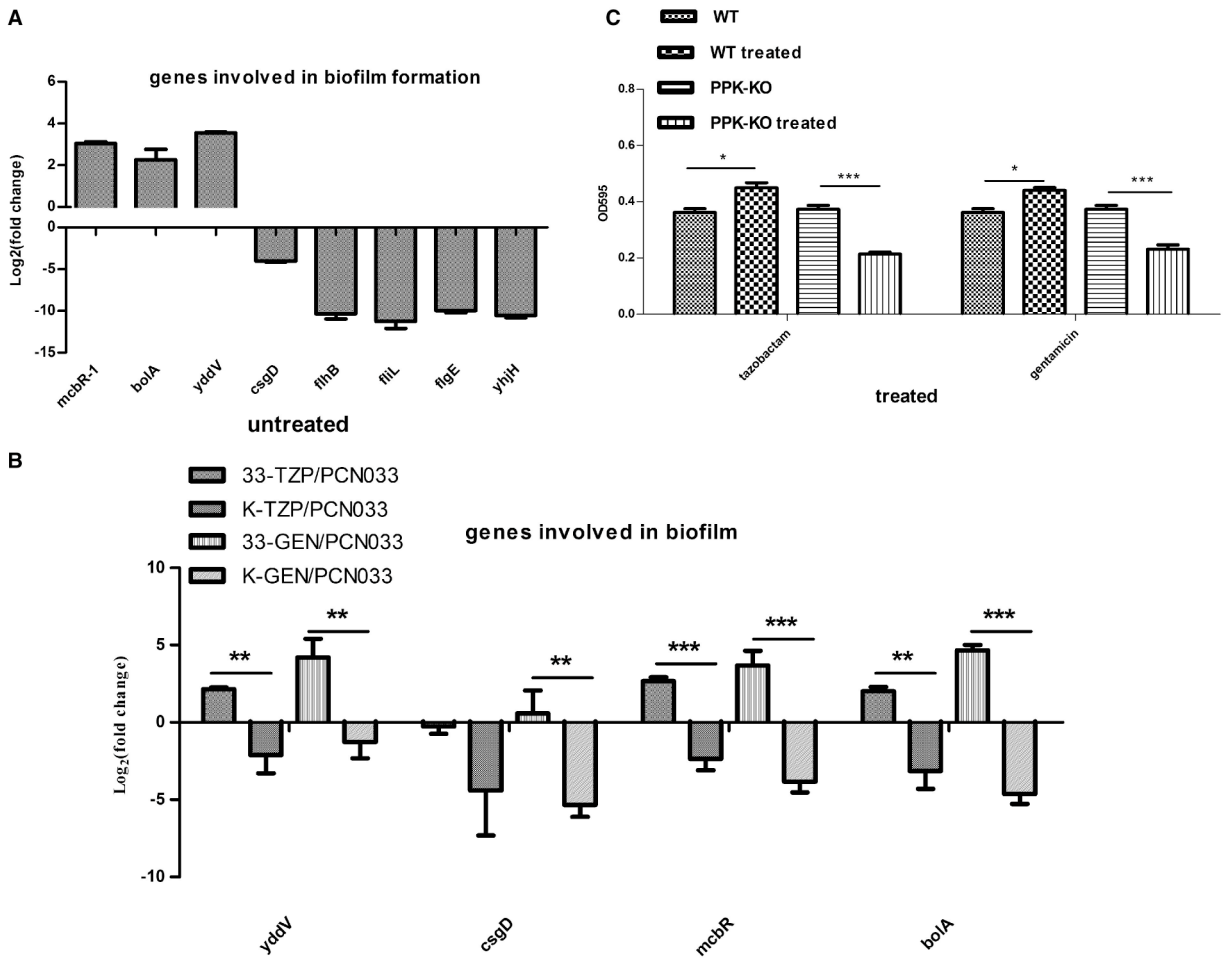

FIGURE 6 | Biofilm formation assay. (A) Validation of the expression of genes involved in biofilm formation. **(B)** Expression levels of genes involved in biofilm regulation during tazobactam and gentamycin treatment. **(C)** Biofilm formation during tazobactam or gentamycin treatment. Expression levels of genes were compared with those in WT (PCN033). 33-TZP/PCN033 indicates the expression levels in PCN033 during tazobactam treatment compared with those in untreated PCN033; K-TZP/PCN033 indicates the expression levels in the Δ*ppk* mutant during tazobactam treatment compared with those in untreated PCN033; 33-GEN/PCN033 indicates the expression levels in PCN033 during gentamycin treatment compared with those in untreated PCN033; K-GEN/PCN033 indicates the expression levels in the Δ*ppk* mutant during gentamycin treatment compared with those in untreated PCN033. WT indicates PCN033 without antibiotic treatment; WT treated indicates PCN033 with antibiotic treatment; PPK-KO indicates the Δ*ppk* mutant without antibiotic treatment; PPK-KO treated indicates the Δ*ppk* mutant with antibiotic treatment. ***$p < 0.000$, **$p < 0.01$, *$p < 0.05$.

cells but decreased in Δ*ppk* planktonic cells when treated with antibiotics.

DISCUSSION

Multidrug-resistant strains of ExPEC present significant challenges to public health and animal husbandry (Girardeau et al., 2003; Johnson et al., 2005; Bergeron et al., 2012). Because pathogenic *E. coli* mainly causes acute infections in its planktonic growth mode (Li et al., 2014), we initially investigated the role of PPK in the antibiotic resistance of ExPEC in the planktonic growth mode. We investigated in detail its susceptibility to different types of antibiotics, mediated by PPK, in *E. coli*. We found that PPK is very important in aminoglycoside tolerance, regulating the expression levels of antibiotic efflux and influx genes in the planktonic growth mode. Our findings indicate that PPK could have utility as a novel antimicrobial drug target.

As reported previously, resistance-conferring proteins and antibiotic efflux and influx porins play important roles in multidrug-resistance. Efflux proteins contribute to antibiotic tolerance by transporting compounds to the extracellular environment, whereas influx proteins have the opposite effect (Wilson, 2014). The expression levels of these genes were determined with RNA-seq, and showed that without antibiotic treatment, they did not differ significantly between WT and the Δ*ppk* mutant. Because PPK is reported to play prominent

roles in the stress responses elicited by other stimuli (Alcántara et al., 2014), we investigated the role of PPK in the antibiotic stress response. Gentamycin and tazobactam were selected to treatthe planktonic cells of WT and the Δ*ppk* mutant. With gentamycin treatment, the expression of the efflux genes *acrA* and *marA* increased more strongly in WT than that in the Δ*ppk* mutant, and the influx porin genes *ompC* and *ompF* decreased more strongly in WT than in the Δ*ppk* mutant. Gentamycin binds the 30S ribosomal subunit and interrupts protein synthesis, thus inhibiting bacterial multiplication (Wargo and Edwards, 2014). According to Gray et al., compounds that interrupt protein metabolism cause intracellular polyP accumulation (Gray et al., 2014). Because it is a high-energy phosphate compound, polyP can be used to phosphorylate the response regulators of two-component systems to regulate gene expression (Sureka et al., 2007). As reported previously, the two-component systems CpxR and BaeR are implicated in antibiotic resistance by regulating the efflux genes of the *acr* operon and *mar* operon (Hu et al., 2011; Weatherspoon-Griffin et al., 2014; Pletzer et al., 2015). We speculated that phosphorylation of BaeR or CpxR using polyP as phosphate donar to modulate expression of *acrA* and *marA* during gentamycin treatment. The expression of porin genes *ompF* and *ompC* is upregulated by cAMP (Dalhoff, 1983), and the level of cAMP is negatively regulated by polyP, which potently inhibits the activity of the class III adenylate cyclases (Guo et al., 2009). Therefore, we speculated that the expression of *ompC* and *ompF* was downregulated by polyP during gentamycin treatment. Therefore, polyP may influence gentamycin tolerance by regulating the expression of antibiotic efflux and influx genes.

With tazobactam treatment, the expression of the resistance gene *bla* and efflux genes *acrA*, *cusC*, and *marA* was upregulated. Tazobactam binds to the periplasmic β-lactamase, and the efflux pump is implicated in resistance to beta-lactams and beta-lactamase inhibitors (Zhanel et al., 2014). However, there are insufficient data to clarify the role of PPK in regulating the expression of efflux pump genes induced by β-lactams. It will be interesting to explore the role of PPK in this process.

Biofilms contribute to antibiotic tolerance and chronic infection; thus, we also investigated the role of PPK in the antibiotic resistance of biofilm-grown cells. We observed that biofilm formation was impaired in the Δ*ppk* mutant when treated with antibiotics. The genes involved in biofilm formation (such as those encoding the fimbriae cluster, flagella cluster, and biofilm regulators BolA and McbR), were downregulated in the Δ*ppk* mutant by both antibiotic treatments. PolyP acts as a "chemical chaperone," stabilizing cytoplasmic proteins intracellularly, similarly to heat shock proteins (Gray et al., 2014), and chaperones are known to be involved in biofilm formation. For example, the chaperone CsgE directs the intracellular localization of CsgA, the major subunit of the extracellular amyloid protein known as curli, which is essential for biofilm formation (Andersson et al., 2013). The FliS protein acts as a chaperone for FliC, a flagellar structural protein that promotes biofilm generation (Xu et al., 2014). The universal heat shock protein chaperones are also implicated in biofilm formation by fungi, such as *Candida albicans* (Robbins et al., 2011; Becherelli et al., 2013), and by Gram-negative bacteria, such as *E. coli* (Grudniak et al., 2013). Ultimately, these different effects reduce biofilm impairment during antibiotic treatment. Interestingly, PPK did not affect the antibiotic tolerance of ExPEC in the biofilm growth mode. Biofilms manifest antibiotic tolerance through many different mechanisms, including preventing the passage of antimicrobial compounds into the cytoplasm and possessing densely adherent growth (Qu et al., 2010). As reported previously, the planktonic and biofilm modes of growth are two distinct bacterial "lifestyles" (Chua et al., 2014), so it will be interesting to explore the roles of PPK in these distinct contexts.

AUTHOR CONTRIBUTIONS

JC and CT designed and supervised the research project. JC wrote the paper, and LS, XW, HC, and TZ revised the manuscript. JC, TZ, and FL performed the experiments. JC and LS processed the data and performed the statistical analysis. All the authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00724

Figure S1 | Percentage activity of different antibiotics in removing biofilm. **(A)** Targeting the cell wall, **(B)** targeting protein biosynthesis, and **(C)** targeting nucleotide metabolism.

Table S1 | Bacteria strains and primers.

Table S2 | Transcription level of genes involved in antibiotic and multi-drug resistance.

Table S3 | Expression of genes implicated in biofilm.

Table S4 | Genes involved in biofilm formation (part results of RNA-seq).

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The Small Colony Variant of *Listeria monocytogenes* Is More Tolerant to Antibiotics and Has Altered Survival in RAW 264.7 Murine Macrophages

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Small Colony Variant (SCV) cells of bacteria are a slow-growing phenotype that result from specific defects in the electron transport chain. They form pinpoint colonies on agar plates and have a variety of phenotypic characteristics, such as altered carbon metabolism, decreased toxin and lytic enzyme production, aminoglycoside resistance, and increased intracellular persistence. They are clinically relevant in *Staphylococcus aureus* and *Pseudomonas aeruginosa*, serving as a reservoir for recurrent or prolonged infections. Here, we found that a SCV mutant in the foodborne pathogen *Listeria monocytogenes* (strain SCV E18), similar to the high persister mutant phenotype, survived significantly better than the wild type when exposed over a 48-h period to concentrations above Minimal Inhibitory Concentration for most tested antibiotics. SCV E18 survived more poorly than the wildtype in unactivated RAW264.7 macrophage cells, presumably because of its reduced listeriolysin O expression, however, it survived better in reactive oxygen species producing, phorbol 12-myristate 13-acetate-activated macrophages. Although SCV E18 was sensitive to oxygen as it entered the stationary phase, it was significantly more tolerant to H_2O_2 than the wild type, which may result from a shift in metabolism, however, further investigation is needed to resolve this. SCV E18 is a spontaneous mutant with a point mutation in the *hemA* gene. A wild type copy of *hemA* was complemented on plasmid pSOG30222, which restored the wild type phenotype. The results reported here suggest that the SCV of *L. monocytogenes* could be of clinical importance and highlight a need for adequate clinical screening for this phenotype, as it could affect antibiotic treatment outcomes.

Keywords: *Listeria monocytogenes*, small colony variants, antibiotic tolerance, persister cells, cell invasion, oxidative stress

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INTRODUCTION

Listeria monocytogenes is a Gram-positive, foodborne pathogen that can cause the rare, but often lethal infection listeriosis. This typically affects pregnant women, neonates, the elderly and the immunocompromised, and can cause mortality rates of up to 34% (Farber and Peterkin, 1991). Upon entry into the gastrointestinal tract, *L. monocytogenes* infects host epithelial cells

and immediately escapes the phagosome where it is free to replicate within the cytosol. Using actin-mediated mobility it spreads to neighboring cells and eventually enters the bloodstream, causing systemic listeriosis if unchecked by the innate immune system (Portnoy et al., 2002). Macrophages are a key part of the innate immune response and control the initial infection by engulfing and killing the bacteria with of a variety of antimicrobial compounds, such as reactive oxygen and nitrogen species (Shaughnessy and Swanson, 2007).

Treatment options for listeriosis are limited as many antibiotics are only bacteriostatic against *L. monocytogenes*. Ampicillin is the treatment of choice, and is sometimes used in combination with gentamicin, although the necessity of this supplemental treatment has been debated (Temple and Nahata, 2000; Mitjà et al., 2009). For patients who cannot tolerate ampicillin, a combination of trimethoprim and sulfamethoxazole, known as co-trimoxazole (SXT), or macrolides are substituted. (Temple and Nahata, 2000). While resistance in *L. monocytogenes* is uncommon, particularly for the clinically relevant antibiotics (Morvan et al., 2010), we recently demonstrated that this bacterium can form multi-antibiotic tolerant persister cells (Knudsen et al., 2013). However, it is not known if such cells are formed *in vivo* during infection, which could further complicate antibiotic treatment.

Persister cells are a very small subpopulation of bacteria that enter a dormant-like state making them refractory to most antibiotics. Their tolerance to antibiotics is not genetically inherited, and can be operationally defined by a biphasic killing curve when treated with bactericidal antibiotics and no observed increase in the Minimum Inhibitory Concentration (MIC) (Lewis, 2010). Persister cells are thought to exist in all bacteria and have been linked to recurrent infections with a number of diseases (Maisonneuve and Gerdes, 2014). Their formation can be either stochastic and the result of phenotypic switching from normally growing cells to those with reduced growth rates (Balaban et al., 2004), or they can be induced by certain conditions, such as biofilms (Lewis, 2010) or internalization by macrophages (Helaine et al., 2014). One of the primary genetic mechanisms of persister formation identified so far are toxin/antitoxin (TA) modules, which consist of a stable toxin that slows bacterial growth or metabolism and an unstable antitoxin that neutralizes the activity of the toxin under growth conditions. By repeatedly exposing *Escherichia coli* to high concentrations of ampicillin, Fridman et al. (2014) found that bacteria can also tolerate antibiotics by extending the lag-phase by mutations in a variety of pathways, essentially rendering the cells dormant until the transient antibiotic pressure is removed.

Another potential reservoir of persistent and recurrent bacterial infections is the so-called Small Colony Variant (SCV) (Kahl et al., 2016). The SCV is a slow growing phenotypic variant that forms pinpoint colonies when plated on agar, and is the result of either thymidine auxotrophy caused by mutations in the thymidylate synthase gene (Besier et al., 2007; Chatterjee et al., 2008) or an interruption in the electron transport chain (ETC), specifically resulting from an absence of menadione or hemin biosynthesis and metabolism. These variants can either be fixed, which is the result of a mutation in one of the biosynthesis or metabolism genes for the aforementioned compounds, or transient, alternating between the wild type and SCV phenotypes during replication (Edwards, 2012). Additional characteristics include altered carbon metabolism, reduced toxin and lytic enzyme production, and resistance to aminoglycosides (Proctor et al., 2014). Aminoglycosides have also been shown to select for the SCV phenotype, as the reduction in the membrane potential resulting from the disrupted ETC will lower the active transport necessary for aminoglycosides to cross the membrane (Balwit et al., 1994). The combination of trimethoprim/sulfamethoxazole (co-trimoxazole) has been found to select specifically for thymidine auxotrophic SCVs due to the anti-folate action of the drug (Garcia et al., 2013). While SCVs have been primarily studied in *Staphylococcus aureus*, they have been described in a number of bacteria including *Pseudomonas aeruginosa*, *Eacherichia coli*, *Vibrio cholera*, *Salmonella*, and *Lactobacillus acidophilus* (Proctor et al., 2006). Furthermore, they have been linked to a number of recurrent infections including those caused by *S. aureus* (Proctor et al., 1995), *P. aeruginosa* (Haussler et al., 1999) and *E. coli* (Roggenkamp et al., 1998; for a comprehensive review see: Kahl et al., 2016). Although typically less virulent due to their slower growth and reduced expression of virulence factors, such as toxins and lytic enzymes, their ability to adhere, invade and persist within the host cell increases, which is thought to be a crucial factor in the ability of SCVs to cause recurrent or persistent infections (Sendi and Proctor, 2009).

We have previously found that exposure to sub-lethal concentrations of triclosan with subsequent selection on gentamicin could generate stable *L. monocytogenes* SCV cells (Christensen et al., 2011), which all had mutations in one of the heme biosynthesis or metabolism genes and exhibited the same traits observed in SCVs of other species (e.g., colony size, decreased haemolytic activity, aminoglycoside resistance and altered carbon utilization). Our SCV E18 strain also showed reduced growth typical of SCVs from other species, taking roughly four more hours to reach the same maximal cell density as the wild type strain ($\sim 10^9$ CFU/ml), however, we also observed a sensitivity to aerated growth conditions (Kastbjerg et al., 2014), which to our knowledge has not been shown in SCVs from other species.

The phenotypic switching of the SCV and the persister cell are thought to be part of a bacterial bet-hedging strategy, allowing a small percentage of the population to survive and repopulate following stress exposure (Sousa et al., 2012). These two phenotypes share a number of other characteristics including slowed growth, intracellular persistence and a link to chronic infections. Thus, given these similarities, we speculate that a stable *L. monocytogenes* heme deficient SCV would show a similar tolerance toward a broad range of antibiotics that persister cells do. Furthermore, we sought to determine if *L. monocytogenes* SCVs are better at invading and surviving intracellularly, as they have been shown to in *S. aureus* (Sendi and Proctor, 2009). Taken together, these results will help to determine the clinical significance of Small Colony Variants in *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Listeria monocytogenes strain N53-1 and a mutant thereof (E18) were included in the present study. N53-1 represents a food-processing plant persistent molecular subtype and was isolated from a fish smokehouse (Wulff et al., 2006). Strain E18 [denoted as strain (1) 1-1 in Christensen et al. (2011)] is a stable SCV of N53-1, with a Single Nucleotide Polymorphism (SNP) in the glutamyl tRNA reductase (*hemA*) gene (Kastbjerg et al., 2014), generated through an adaptive evolution experiment where N53-1 was exposed to sub-lethal levels of triclosan and subsequently selected on gentamicin (Christensen et al., 2011). Strains were stored at −80°C and grown on Brain Heart Infusion (BHI; Oxoid CM 1135) agar at 37°C for 24 h. Overnight cultures were achieved by inoculating single colonies in 10 ml of BHI broth (Oxoid CM 1135) and incubating at 37°C with shaking at 250 rpm.

Preparation of Antibacterial Agents

Fresh antibiotic solutions were prepared for each experiment: ampicillin (dissolved in sterile MilliQ water; Sigma–Aldrich A9518), erythromycin (dissolved in 96% ethanol; Sigma–Aldrich E6376), gentamicin (dissolved in sterile MilliQ water; Sigma–Aldrich G3632), norfloxacin (dissolved in sterile MilliQ with 1% glacial acetic acid; Fluka N9890) vancomycin (dissolved in sterile MilliQ; Sigma–Aldrich V2002) and co-trimoxazole, which is comprised of one part trimethoprim (dissolved in sterile MilliQ with 1% glacial acetic acid; Sigma–Aldrich 92131) and five parts sulphamethoxazole (dissolved in acetone; Fluka S7507). H_2O_2 (30% in water; Sigma–Aldrich 216763) was diluted to 20 mM in sterile MilliQ water.

Minimal Inhibitory Concentration (MIC) Assay

Antibiotic MIC values were determined in BHI broth as previously described (Cockerill et al., 2012). In brief, stationary phase cultures of N53-1 and SCV E18 were adjusted to $OD_{600} = 0.2$ and further diluted 1000-fold, corresponding to a CFU/ml of 10^5. The adjusted cultures were tested against norfloxacin, ampicillin, gentamicin, and co-trimoxazole (trimethoprim/sulfamethoxazole) using a twofold dilution series of concentrations ranging from 100 to 0.10 μg/ml in 96-well plates (Thermo Scientific 163320) and incubated at 37°C. MIC for N53-1 and SCV E18 was determined by visual inspection after 24 and 48 h of incubation at 37°C, respectively. Two biological replicates were performed.

Killing Kinetic Assays

To compare the antibiotic and H_2O_2 sensitivity of SCV E18 and N53-1, killing kinetics were performed according to Knudsen et al. (2013). Overnight cultures were diluted 10^6-fold in 10 ml BHI broth and incubated into early stationary phase for 16 h at 37°C with shaking at 250 rpm. Each culture was then adjusted to an optical density at 600 nm (OD_{600}) of 0.2 in 2 ml BHI broth and challenged with antibiotic (30X MIC except nitrofurantoin, which was 10X MIC) at 37°C. Bacterial counts were performed at 0, 4, 10, 24, 48, and 72 h. The antibiotics and concentrations used were norfloxacin (100 μg/ml), ampicillin (185 μg/ml), erythromycin (6 μg/ml), vancomycin (47 μg/ml), and co-trimoxazole (95 μg/ml). Cultures treated with H_2O_2 were grown and adjusted as described above, then treated with 20 mM and incubated under stagnant conditions at 37°C for 2 h. Three independent biological replicates were performed for each experiment and the limit of detection was 10^2 CFU/ml.

Anaerobic Culturing Conditions

To verify that E18 was sensitive to oxygen, it, along with N53-1, were cultured in anaerobic conditions. Oxygen was removed from the media by autoclaving, followed by incubation in a HP0011 anaerobic jar (Oxoid) with an anaerobe gas generation bag (Sigma–Aldrich 68061) for 24 h. Ten microliters of an overnight culture was added to the anaerobic BHI, along with a new anaerobe gas generation bag and incubated on the lab bench for 3 h to allow for an anaerobic atmosphere to be generated before incubation with shaking. The cultures were grown for 72 h at 37°C with shaking at 200 rpm. Two biological replicates were performed.

hemA Complementation

E18 carries several single nucleotide polymorphisms (SNPs) of which one is in the *hemA* gene (Kastbjerg et al., 2014). To verify the role of the SNP in *hemA* in the SCV E18 strain, a *hemA* complementation strain was constructed. The *hemA* gene with a 128 bp upstream region including the native promoter was amplified from genomic DNA of N53-1 with the forward primer 5′-AAACTCGAGTCATCCGTTAACTCCTCG and the reverse primer 5′-AAAGAATTCATAGAAGGAGTTGGAATGGA, which contained terminal XhoI and EcoRI sites, respectively, using Phusion High Fidelity DNA Polymerase (NEB, M0530S). The pSOG30222 plasmid (Hain et al., 2008) and *hemA* amplicon were digested with XhoI and EcoRI, and ligated overnight using a 3:1 insert to vector ratio, then electroporated into 60 μl of electrocompetent DH5α cells using a MicroPulser (BioRad 165-2100). Transformants were selected on Luria–Bertani plates with 100 μg/ml erythromycin and verified by colony PCR and sequencing (GATC Biotech). Plasmids containing the correct pSOG30222::*hemA* construct, along with an unmodified pSOG30222 control plasmid, were transformed into the SCV E18 strain. Transformants were selected on BHI agar with 5 μg/ml erythromycin and confirmed with colony PCR and sequencing. With the exception of the killing kinetic assay, all complementation experiments were carried out in the presence of 5 μg/ml erythromycin. Colony pictures were taken of each strain with an Olympus BX51 microscope at 40× magnification.

Macrophage Internalization and Survival Assay

A murine macrophage cell line, RAW 264.7 (Sigma–Aldrich 91062702), was used to test bacterial internalization and intracellular survival using a protocol adapted from Bateman and Seed (Bateman and Seed, 2012). RAW 264.7 macrophages were grown in Dulbecco's Modified Eagle's Medium – high glucose

(Sigma–Aldrich D6546), 10% heat inactivated fetal bovine serum (Sigma–Aldrich F9665) and L-glutamine (Sigma–Aldrich G7513) in 150cc tissue culture flasks, for 10–15 passages prior to use. A cell scraper was used to disjoin the adherent cells from the surface. RAW 264.7 cells were seeded into 24-well plates at a density of 2×10^6 cells/ml in RAW 264.7 culture media and then incubated for a total of 48 h at 37°C with 5% CO_2. During the 48 h, one group of RAW 264.7 cells was stimulated for 18 h with 1 ng/ml IFNγ, required for macrophage activation, and a second group was stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA), a known inducer of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (Walloschke et al., 2010) for 2 h, while a third control group was left unstimulated. Prior to infection, IFNγ and PMA were removed from the RAW 264.7 containing wells. Overnight cultures of N53-1 and SCV E18 were diluted 10^6-fold in 10 ml of BHI media and grown at 37°C for 16 h. *L. monocytogenes* cultures were adjusted to a density of 2×10^7 CFU/ml in PBS and added to the confluent RAW 264.7 containing wells, achieving a multiplicity of infection (MOI) of 10. Plates were centrifuged for 5 min at 1,000 rpm to synchronize the infection and incubated at 37°C for 1 h. The plates were then incubated for an additional 2 h in media containing 100 μg/ml gentamicin, which was reduced to 50 μg/ml gentamicin for the remainder of the incubation. In order to verify that gentamicin did not favor the resistant SCV E18 during the 21 h incubation step, a control experiment was performed using the same methods as the unactivated macrophage experiment, with the exception that the media with 50 μg/ml gentamicin was substituted with PBS. To enumerate the surviving *L. monocytogenes* per well, RAW 264.7 cells were washed twice with PBS, followed by lysis with 0.1% Triton-X 100 (Sigma–Aldrich T8787) and then serial dilutions of the intracellular bacteria were plated on BHI agar plates. Internalization was measured following 3 h of incubation, while survival was measured after 24 h.

Statistical Analysis

CFU/ml values for each biological replicate were $\log_{10}$ transformed prior to statistical analysis. For the killing kinetic and O_2 sensitivity assays, significant differences between the N53-1 and SCV E18 were determined using a paired *t*-test, where significance is equal to $P < 0.05$. Significance between N53-1 and SCV E18 for the RAW 264.7 internalization and survival assays was determined using a paired *t*-test ($P < 0.05$) with the $\log_{10}$ transformed CFU/ml values. Significance for each strain between the RAW264.7 treatment groups was calculated using a one-way ANOVA test for the $\log_{10}$ transformed CFU/ml values, where $P < 0.05$ determined significance. Significance between the two strains within each RAW264.7 treatment group was calculated using a paired *t*-test ($P < 0.05$). The limit of detection for all CFU/ml values was 10^2. All data analysis was performed in Excel. Replicates falling below the detection limit were set to 99 for statistical analysis.

RESULTS

SCV E18 Shows Increased Tolerance to Antibiotics

The *L. monocytogenes* SCVs, selected on gentamicin, were resistant to other aminoglycosides (Kastbjerg et al., 2014). To determine if the SCV phenotype conveys resistance to other antibiotics, MIC values of selected antibiotics were tested for both N53-1 and SCV E18, which were found to be the same for the two strains (**Table 1**).

Because *L. monocytogenes* SCV grow slower than the wild type (Kastbjerg et al., 2014), and because of the lowered oxidative phosphorylation observed in other SCV organisms, such as *S. aureus* (Proctor et al., 1998), we hypothesized that *L. monocytogenes* SCVs would have an increased tolerance to other classes of antibiotics, which was evaluated using time dependent killing experiments. With the exception of erythromycin, the SCV E18 strain survived significantly better for each antibiotic challenge up to 48 h (**Figure 1**; ampicillin, $P = 0.0007$; co-trimoxazole, $P = 0.02$; vancomycin, $P = 0.02$ and norfloxacin, $P = 0.03$), with between one and threefold higher $\log_{10}$ CFU/ml as compared to the N53-1. When treated with norfloxacin, both N53-1 and SCV E18 exhibited a biphasic killing curve with a rapid decline to approximately three and five $\log_{10}$ CFU/ml, respectively, which remained stable throughout the 72 h experiment (**Figure 1A**). Ampicillin has a delayed bactericidal effect on *L. monocytogenes* (Winslow et al., 1983), which was also observed with SCV E18, as is evidenced by the roughly three-fold $\log_{10}$ reduction in CFU/ml over the 48–72 h time points (**Figure 1B**). However, this delayed bactericidal effect of ampicillin was not observed for N53-1 over the course of the

TABLE 1 | Minimum Inhibitory Concentration (MIC) values for *Listeria monocytogenes* wild type strain N53-1 strain and SCV E18 strain.

	MIC μg/ml					
Strain	**Ampicillin**	**Co-trimoxazole**	**Norfloxacin**	**Erythromycin**	**Vancomycin**	**Gentamicin**
N53-1	3.13	0.78	3.13	0.20	1.56	0.40
E18	3.13	0.78	3.13	0.20	1.56	12.50
E18 pSOG30222::*hemA*	1.56	0.78	3.13	–	1.56	0.40
E18 pSOG30222	1.56	0.78	3.13	–	1.56	12.50

The MIC assay was performed using BHI broth and incubated at 37°C for 24 and 48 h for N53-1 and SCV E18, respectively. All experiments were done with two independent replicates. – , not tested.

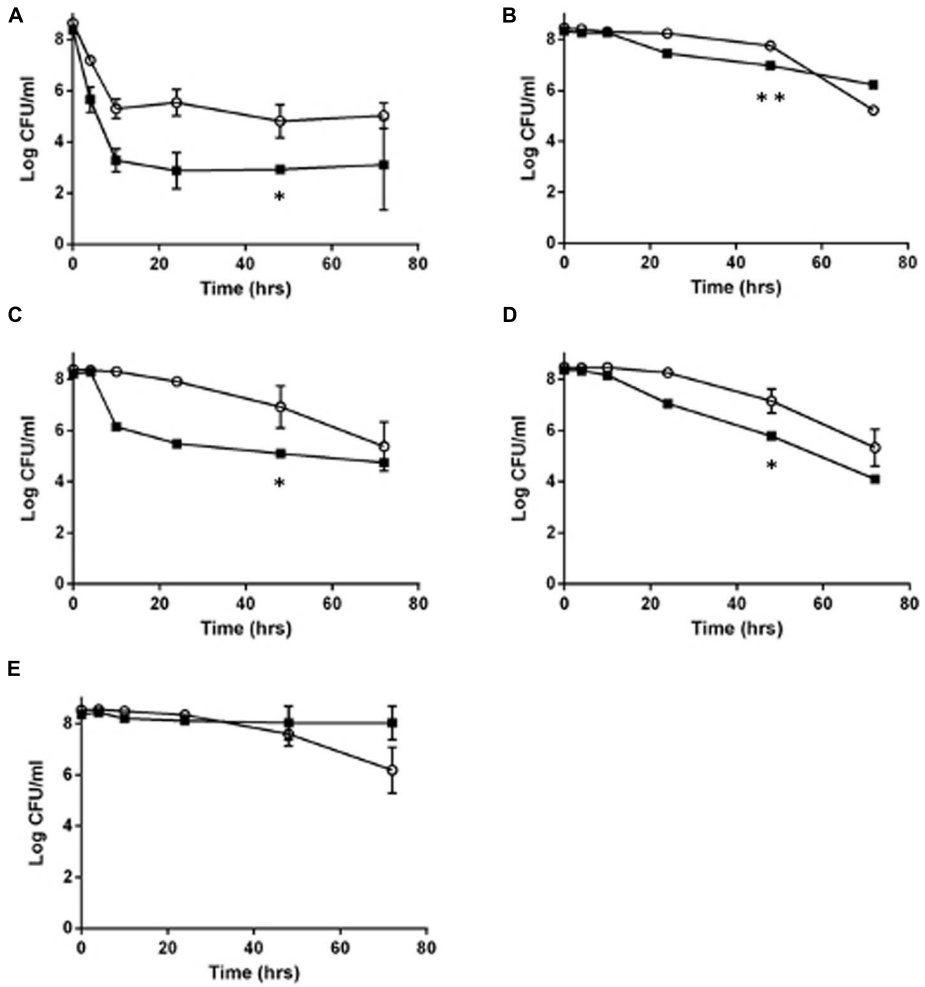

FIGURE 1 | Killing of *Listeria monocytogenes* N53-1 (■) and SCV E18 (○) over the course of 72 h under shaking conditions with (A) norfloxacin, (B) ampicillin, (C) co-trimoxazole, (D) vancomycin, and (E) erythromycin. Error bars represent standard deviation of the mean for three biological replicates. Significance at the 48-h time point was determined using a paired *t*-test (*$p < 0.05$ and **$p < 0.005$).

72 h killing kinetic (**Figure 1B**). By the end of the 72 h treatment with co-trimoxazole, SCV E18 decreased in a linear manner to a comparable level with N53-1 (2.3×10^5 and 5.6×10^4 CFU/ml, respectively; $P = 0.45$; **Figure 1C**). Vancomycin had an initial bacteriostatic effect on both strains, followed by linear killing, however, the bactericidal effect began earlier for N53-1 ($T = 10$), while the SCV E18 strain resisted killing until the 24 h time point (**Figure 1D**). Erythromycin was bacteriostatic on both strains up to the 48 h time point, however, bacterial counts for SCV E18 decreased after 48 h, while the N53-1 remained stable (**Figure 1E**).

While the SCV phenotype in *L. monocytogenes* does not confer notable resistance to antibiotics besides the aminoglycosides, they survived significantly better under high antibiotic concentrations for up to 48 h under ampicillin or co-trimoxazole, and up to 72 h when exposed to norfloxacin or vancomycin. In contrast, by 72 h of exposure to erythromycin or ampicillin, the SCV has fewer CFU/ml than the wild type.

Oxygen Sensitivity, But H_2O_2 Resistance, of the SCV E18

When grown under shaking conditions, SCV E18 grows to a maximum density of approximately 1×10^9 CFU/ml after 12 h, where after the CFU/ml decreases rapidly, eventually dropping below the limit of detection after 24 h (Kastbjerg et al., 2014). Here, we grew cultures of the SCV E18, along with the N53-1, anaerobically to test if the observed decrease in CFU/ml is caused by exposure to oxygen. When grown anaerobically, cultures of the SCV E18 grow to higher densities (8.1×10^8 CFU/ml) after 72 h, than the N53-1 strain (2.5×10^8 CFU/ml; $P = 0.002$) (**Table 2**), suggesting that oxygen is toxic to the SCV E18 strain. Under anaerobic growth conditions the SCV E18 strain retained the pin point colony morphology when plated, whereas the N53-1 strain exhibited variable colony size.

To test if the oxygen sensitivity was related to a general deficiency in the oxidative stress response, such as the absence of catalase in the SCV E18, we treated with 20 mM H_2O_2

TABLE 2 | Oxidative stress sensitivity of *L. monocytogenes* N53-1 and SCV E18 as measured by aerobic and anaerobic growth, and exposure to 20 mM H_2O_2 for 2 h.

	Bacterial count (Log_{10} CFU/ml)			
	Growth in BHI for 72 h		Survival in 20 mM H_2O_2	
	Aerobic	Anaerobic	0 h	2 h
N53-1	9.4 ± .01	8.4 ± 0.03	7.2 ± 0.04	3.7 ± 0.09
E18	BD	8.9 ± 0.02	7.2 ± 0.13	6.3 ± 1.02
E18 pSOG30222::*hemA*	8.1 ± 0.09	–	8.0 ± 0.22	5.2 ± 0.17
E18 pSOG30222	BD	–	8.0 ± 0.06	6.5 ± 0.41

All experiments are log_{10} CFU/ml mean values of three biological replicates ± standard deviation. BD, below the limit of detection; –, not tested.

for 2 h. Surprisingly, the SCV E18 survived significantly better ($P = 0.008$) than the wild type, with the number of colony forming bacteria following treatment being 33% of the inoculum, whereas the N53-1 strain survived at a rate of 0.03% (**Table 2**). Thus, similar to the increased tolerance toward antibiotics, and regardless of the oxygen sensitivity and lack of catalase (Kastbjerg et al., 2014), the SCV E18 strain exhibits a greater ability to resist killing by H_2O_2 over the wild type.

hemA Complementation Restores Wild Type Phenotype of SCV E18

By genome sequencing and analyses, we previously demonstrated that all of the *L. monocytogenes* SCVs had several mutations, including a mutation in the *hemA* gene (Kastbjerg et al., 2014). To determine if the tested aspects of the SCV phenotype were due to the SNP in the *hemA* gene, a wild type *hemA* complemented strain of E18 was constructed. This complemented strain regained the large colony phenotype (**Figure 2A**), had restored gentamicin sensitivity identical to the N53-1 strain (**Table 1**), regained sensitivity to H_2O_2 and lost its sensitivity to oxygen, as demonstrated by the presence of viable colonies after culturing for 72 h (**Table 2**). Furthermore, the level of antibiotic tolerance was evaluated with a time dependent killing experiment using norfloxacin. The killing curve of the complemented *hemA* strain was significantly different from the empty vector control ($P = 0.0000046$), and was similar to the killing of N53-1 strain, while the empty vector control showed a very similar curve to its non-transformed counterpart SCV E18 (**Figure 2B**).

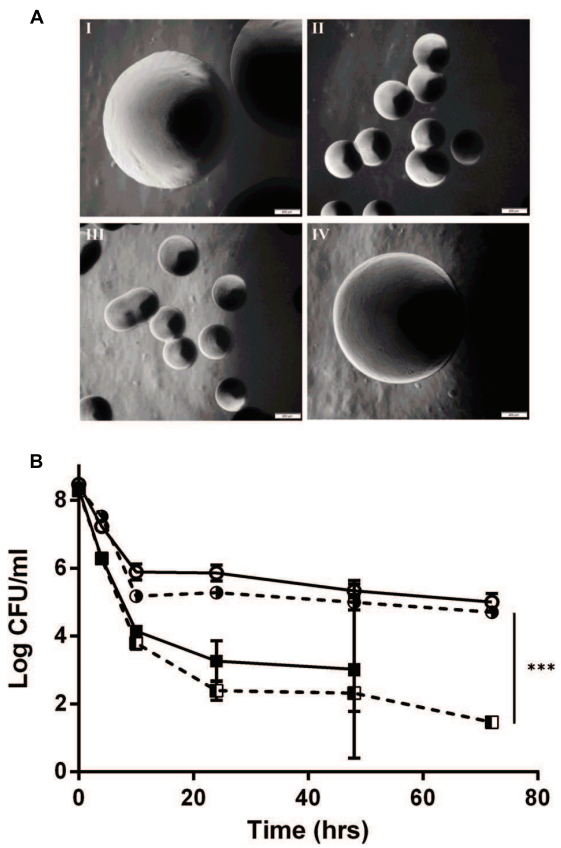

FIGURE 2 | *hemA* complementation experiments showing (A) colony morphology at 40x magnification of I: N53-1, II: SCV E18, III: E18 pSOG30222, and IV: E18 pSOG30222::*hemA* after 24 h of incubation at 37°C on BHI plates and (B) Killing of *L. monocytogenes* N53-1 (■) and SCV E18 (○) strains, as well as the *hemA* complemented (◧) and empty vector control E18 (◑) strains exposed to 100 μg/ml norfloxacin for 72 h. The data represents three biological replicates with standard deviation of the mean. Significance was determined using a paired *t*-test (***$p < 0.000005$).

SCV E18 Is Less Affected by Macrophage Status Than Wild Type N53-1

We investigated the survival ability of the wild type and the SCV E18 in the IFNγ activated and un-stimulated macrophage model. Additionally, due to the increased tolerance of SCV E18 toward H_2O_2, we used macrophages stimulated with PMA, a known activator of the NADPH oxidase pathway. Three hours following infection of the un-stimulated macrophage there was a high degree of internalization and intracellular growth of N53-1 and SCV E18, with 7.6 log_{10} CFU/ml and 7.5 log_{10} CFU/ml recovered, respectively, a difference that was not statistically significant ($P = 0.77$). These internalization findings were similar for the other two treatment groups (**Figure 3A**). By 24 h of incubation in the un-stimulated macrophage, bacterial counts were drastically reduced for both strains, although N53-1 survived significantly better ($P = 0.006$) with 3.6 log_{10} CFU/ml of the N53-1 and 2.3 log_{10} CFU/ml for SCV E18 (**Figure 3B**). When macrophages

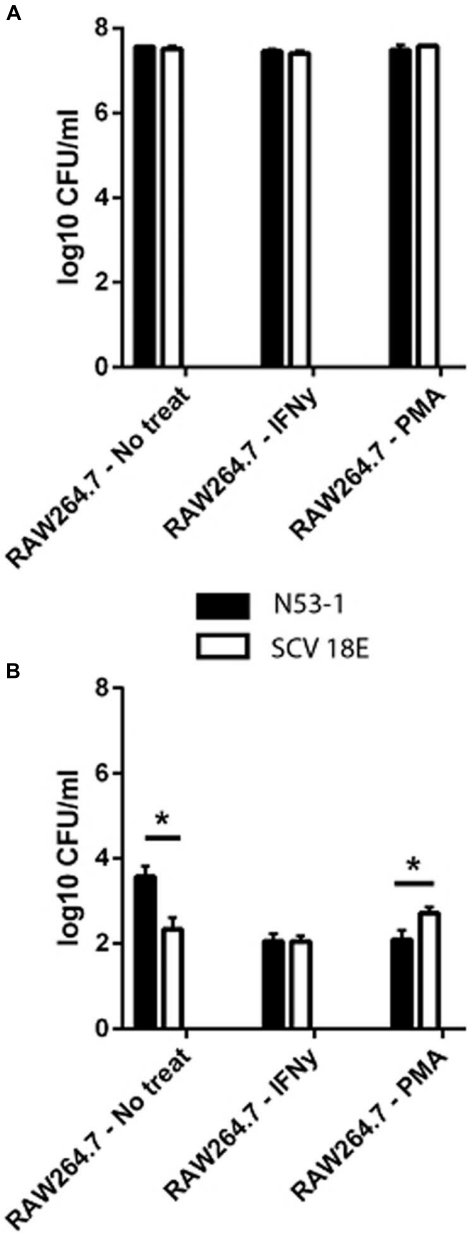

FIGURE 3 | Intracellular (A) internalization and (B) survival of *L. monocytogenes* N53-1 and SCV E18 in RAW264.7 cells treated with IFNγ, PMA and no treatment using a multiplicity of infection (MOI) of 10. Bacterial counts for the internalization assay were determined after 2 h of 100 μg/ml gentamicin exposure. Survival was determined after 21 additional hours of incubation with 50 μg/ml gentamicin. Values are displayed as the $\log_{10}$ transformed mean of three biological replicates with standard deviation. Significance was determined using a paired *t*-test (*$p < 0.05$).

were activated with IFNγ, the survival difference between strains vanished, as the number of recovered N53-1 bacteria at 24 h was reduced to 2.06 $\log_{10}$ CFU/ml, while the SCV E18 burden was almost unchanged by IFNγ activation (2.15 $\log_{10}$ CFU/ml) (**Figure 3B**). Increased reactive oxygen species (ROS) production, via PMA activation of the NADPH oxidase pathway, resulted in a slight, but significantly higher bacterial burden of macrophages infected with SCV E18 (2.7 $\log_{10}$ CFU/ml), than N53-1 (2.1 $\log_{10}$ CFU/ml; $P = 0.02$) (**Figure 3B**). While activation of macrophages with IFNγ or stimulation with PMA significantly reduced the intracellular bacterial load of N53-1 ($P = 0.01$), the SCV E18 was not sensitive to either treatment and remained unchanged across groups ($P = 0.17$).

To summarize, macrophages were equally poor at controlling the bacterial burden of both N53-1 and SCV E18 in the short term ($T = 3$ h), however, by 24 h the CFU/ml for all intracellular bacteria had been severely reduced. While survival in the un-activated macrophage was attenuated for SCV E18, as compared to N53-1, it was unaffected by the status of the macrophage unlike N53-1, which was significantly reduced in CFU/ml upon macrophage activation with IFNγ or stimulation with PMA.

DISCUSSION

The Small Colony Variant (SCV) has been described for decades in several bacteria such as *S. aureus*, *E. coli* and *P. aeruginosa* (Proctor et al., 2006). However, they were only recently identified in *L. monocytogenes* and, with the exception of aminoglycoside resistance (Kastbjerg et al., 2014), their importance with respect to the treatment of listeriosis has not been investigated. This study is the first to demonstrate that the SCV phenotype of *L. monocytogenes* caused by a single point mutation in the *hemA* gene has the potential to complicate treatment by causing an increase in tolerance toward most of the clinically relevant antibiotics.

As shown previously (Kastbjerg et al., 2014), SCV E18 exhibited resistance to the aminoglycoside gentamicin, a defining trait of the electron-transport deficient SCV (Proctor et al., 2006). This is particularly relevant for listeriosis, as gentamicin is the most common secondary antibiotic used for treatment (Temple and Nahata, 2000). While the SCV E18 showed no increase in MIC for the other antibiotics tested, indicating a lack of acquired resistance, it was nonetheless able to survive lethal concentrations of norfloxacin, ampicillin (up to 48 h), co-trimoxazole, and vancomycin significantly better than the wild type, which taken together, are the definition of antibiotic tolerance (Dimitrijovski et al., 2015). The increase in tolerance toward vancomycin, second generation fluoroquinolones (e.g., norfloxacin) and beta-lactams (e.g., ampicillin) has been observed with *S. aureus* SCVs and is presumably the result of their slow growing SCV phenotype (Chuard et al., 1997; Garcia et al., 2012; Lechner et al., 2012). Antibiotic tolerance poses a unique clinical dilemma, as any testing on isolates relying solely on MIC values could obscure the true antimicrobial susceptibility of the SCV and lead to sub-optimal treatment strategies.

In contrast to the other antibiotics, SCV E18 did not survive better under erythromycin pressure at any time point during the experiment and, as with ampicillin, became more sensitive after 48 h of killing. This could indicate that whatever protective effect the SCV phenotype contributes toward other antibiotics does not apply to the macrolide class, which target protein synthesis. Interestingly, *S. aureus* SCVs were not isolated from cystic fibrosis

patients treated exclusively with the macrolide azithromycin, whereas when treated with other antibiotics, SCVs were observed in 46% of patients colonized with *S. aureus* (Kahl et al., 2003; Green et al., 2011). Although the SCV *L. monocytogenes* E18 tolerates ampicillin better than the wild type N53-1 in the short term, the sensitivity toward extended exposure (as evidenced by the pronounced decrease in bacterial count following 48 h of treatment) and the fact that long-term (between 2 and 8 weeks) ampicillin therapy is the primary treatment of choice for listeriosis (Temple and Nahata, 2000), could explain why the SCV phenotype has, to our knowledge, not been observed in in clinical cases. However, it is also possible that current screening procedures in the clinical laboratory are inadequate, leading the SCV sub-population to go unnoticed (Proctor et al., 2006). The slower growth of SCV E18 would likely cause it to be overshadowed in a mixed culture containing the wild type. Furthermore, the altered sugar metabolism and lack of catalase observed in SCV E18 (Kastbjerg et al., 2014), as well as the fact that some of the media used to grow *L. monocytogenes* contain the compounds SCVs are auxotrophic for, would complicate proper identification of any SCV *L. monocytogenes* clinical isolates.

We were expecting the SCV E18 to be more sensitive to H_2O_2 because it does not produce catalase, however, it was able to better withstand H_2O_2 than the wild type. As H_2O_2 reacts with the heme cofactor of proteins (Nagababu and Rifkind, 1998), our observation may simply be the result of fewer targets in the heme deficient SCV E18. Alternatively, tolerance toward H_2O_2 may be due to a change in the metabolism of the SCV, as suggested by Painter et al. (2015). In addition to showing that heme auxotrophic SCVs of *S. aureus* were more tolerant of H_2O_2, they added 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO), which can induce the SCV phenotype by blocking the electron transport chain of Gram-positive bacteria, to wild type *S. aureus* either hours or minutes before exposure to lethal levels of H_2O_2. Those bacteria grown for hours in the presence of HQNO survived the H_2O_2 exposure significantly better than the wild type, whereas those that received the HQNO only minutes before did not. This demonstrates that the tolerance is not simply the result of reduced electron transport, but as the authors suggest, a global physiological change caused by the loss of the electron transport chain (e.g., a switch to anaerobic metabolism), which could also explain our similar observation in *L. monocytogenes*.

In addition to the typical characteristics of the SCV, *L. monocytogenes* SCVs exhibit a unique sensitivity to growth under shaking conditions as they enter the stationary phase (Kastbjerg et al., 2014). Here, we demonstrate that this results from the presence of oxygen in the media. The addition of catalase, which converts H_2O_2 to water and oxygen, or heme (necessary for the biosynthesis of catalase) to the growth media abolished this effect, suggesting that the oxygen sensitivity is due to the inability of the heme-deficient SCV mutant to produce catalase (Kastbjerg et al., 2014). However, to our knowledge, this oxygen sensitivity has not been observed in heme-deficient SCVs of other catalase-dependent species (Roggenkamp et al., 1998; Zahra et al., 2013), and this fails to explain why the associated toxicity only manifests in the stationary phase. Few explanations seem to coincide with the observed increased tolerance toward H_2O_2 and extended viability of SCV E18 cells when treated with antibiotics, however, we believe this is an important observation that warrants further research.

When the wild type and SCV E18 were inoculated with RAW264.7 murine macrophages over the course of 3 h, we observed no difference between the internalization of the two strains. However, prolonged incubation of the infected macrophages revealed differences between the fates of each strain depending on the activation status of the macrophages. N53-1 survived better than SCV E18 in the naïve macrophages, however, this difference disappeared once macrophages were activated with IFNγ where the wild type was reduced to the same CFU/ml as the mutant. The former observation is likely explained by the reduced expression of the pore-forming toxin Listeriolysin O (LLO) (Kastbjerg et al., 2014) in SCV E18, which has been shown to be produced in murine macrophages (Moors et al., 1999) and is necessary for *L. monocytogenes* to escape from the phagosome (Bielecki et al., 1990). The latter observation corresponds with studies showing that IFNγ-activated macrophages are able to block the escape of *L. monocytogenes* (Portnoy et al., 1989) by a process thought to be mediated by ROS inactivation of the LLO toxin (Myers et al., 2003). This suggests that macrophages need not first be activated by IFNγ in order to control *L. monocytogenes* SCVs, which contrasts to SCVs from other species, and could explain the lack of observed clinical *L. monocytogenes* SCVs. On the other hand, LLO is the most immunogenic antigen for the T cell response, which mediates the ultimate removal and adaptive immunity to *L. monocytogenes* (Vijh and Pamer, 1997), thus a reduction in LLO expression may lead to a weaker adaptive immune response to SCV E18 and could explain the observation that mutants incapable of producing LLO persist for significantly longer in the bone marrow of infected mice (Hardy et al., 2009). Furthermore, the PMA stimulated macrophage showed that SCV E18 is significantly better at surviving the bactericidal effects of ROS within the phagosome, which is consistent with its observed increase in H_2O_2 tolerance, and could have implications on the fate of SCV *L. monocytogenes* in neutrophils that rely predominantly on NADPH oxidase for their bactericidal activity (Segal, 2005).

Our complementation experiment showed that this phenotype can arise through a single base-pair substitution in the *hemA* gene, and given the prevalence of verified SCV selection pressures *L. monocytogenes* faces both in the environment and during treatment (e.g., triclosan, gentamicin and co-trimoxazole), the probability of exposure to SCV *L. monocytogenes* seems high. Therefore, it is surprising that no clinical cases of SCV associated listeriosis have been reported. Perhaps the long treatment with ampicillin is enough to eradicate the SCV, or the immune system is better able to control SCV *L. monocytogenes*, as indicated by our findings with non-IFNγ activated RAW264.7 murine macrophages. However, it seems equally likely that this elusive phenotype has gone unnoticed due to a lack of adequate screening procedures in clinical laboratories.

Much like persister cells, SCVs have also been shown to revert to the wildtype phenotype. When clinical and laboratory strains of *S. aureus* were cyclically grown with and without gentamicin, an identical proportion of SCVs upon each

exposure to the antibiotic was observed (Massey et al., 2001). Drenkard and Ausubel (2002) found the same phenotypic switching in *P. aeruginosa*, and also identified a protein that modulates the switch between the wild type and SCV phenotypes. This phenotypic heterogeneity, often referred to as a bet-hedging strategy, allows a portion of the bacterial population to survive a stress (e.g., antibiotic exposure or the phagosome), without giving up any fitness advantages associated with mutation. It is plausible that the majority of SCVs *in vivo* are the result of phenotypic switching, and although, we observed no reversion with SCV E18, this mutant and others like it could represent a small subset of SCVs locked in the on position. This would add another treatment challenge, as the bacterial population as a whole would be protected from antibiotics, while simultaneously capable of full virulence (Tuchscherr et al., 2011). Reports of SCVs from a variety of bacterial species being linked to persistent and chronic infections are numerous (Proctor et al., 1995; von Eiff et al., 1997; Roggenkamp et al., 1998; Seifert et al., 1999; Häussler et al., 2003), and while uncommon, cases of recurrent listeriosis have been reported and the cause of these treatment failures were not determined (McLauchlin et al., 1991; Sauders et al., 2001; Kleemann et al., 2009).

Although *L. monocytogenes* SCVs may be unique in that macrophages are able to control them without first becoming activated by IFNγ, they may yet be able to better survive other aspects of the immune system, and the presence of an easily generated, multi-antibiotic tolerant sub-population is nonetheless a cause for concern. Awareness and adequate screening for SCVs in clinical isolates, such as extended incubation times and inclusion of isolates with non-standard sugar metabolism or those lacking catalase activity, would also help to determine the role (if any) this phenotype plays in human infection with *L. monocytogenes*, and we believe the evidence presented here warrants such measures being employed in clinical laboratories.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: TC, LG, and GK. Performed the experiments: TC. Analyzed the data: TC, LG, and GK. Wrote the manuscript: TC, LG, and GK.

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Carriage of Extended-Spectrum Beta-Lactamase-Plasmids Does Not Reduce Fitness but Enhances Virulence in Some Strains of Pandemic *E. coli* Lineages

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Pathogenic ESBL-producing *E. coli* lineages occur frequently worldwide, not only in a human health context but in animals and the environment, also in settings with low antimicrobial pressures. This study investigated the fitness costs of ESBL-plasmids and their influence on chromosomally encoded features associated with virulence, such as those involved in the planktonic and sessile behaviors of ST131 and ST648 *E. coli*. ESBL-plasmid-carrying wild-type *E. coli* strains, their corresponding ESBL-plasmid-"cured" variants (PCV), and complementary ESBL-carrying transformants were comparatively analyzed using growth curves, Omnilog® phenotype microarray (PM) assays, macrocolony and biofilm formation, swimming motility, and RNA sequence analysis. Growth curves and PM results pointed toward similar growth and metabolic behaviors among the strains. Phenotypic differences in some strains were detected, including enhanced curli fimbriae and/or cellulose production as well as a reduced swimming capacity of some ESBL-carrying strains, as compared to their respective PCVs. RNA sequencing mostly confirmed the phenotypic results, suggesting that the chromosomally encoded *csgD* pathway is a key factor involved. These results contradict the hypothesis that ESBL-plasmid-carriage leads to a fitness loss in ESBL-carrying strains. Instead, the results indicate an influence of some ESBL-plasmids on chromosomally encoded features associated with virulence in some *E. coli* strains. In conclusion, apart from antibiotic resistance selective advantages, ESBL-plasmid-carriage may also lead to enhanced virulence or adaption to specific habitats in some strains of pandemic ESBL-producing *E. coli* lineages.

Keywords: ESBL-producing *E. coli*, ESBL-plasmids, fitness costs, enhanced virulence, biofilm formation, plasmid and host interaction

INTRODUCTION

The global emergence of antimicrobial resistance, including extended-spectrum beta-lactamases (ESBL), is driven not only by plasmids encoding for these factors, but is also crucially influenced by pandemic bacterial clonal lineages (Naseer and Sundsfjord, 2011). The success of the pathogenic ESBL-producing *E. coli* clonal lineage of sequence type ST131 and virulence-associated phylogenetic group B2 is particularly noteworthy (Nicolas-Chanoine et al., 2014). B2-ST131 is found worldwide in environments with high antimicrobial selection pressures, including human and veterinary clinics and communities (Nicolas-Chanoine et al., 2008; Ewers et al., 2010). ST131 is also found in remote areas and wildlife (Bonnedahl et al., 2014), where antimicrobial influence is thought to be of lower importance. Despite the recognition of the human clinical reservoir as most abundant, several studies have demonstrated increasing prevalence of ST131 in animals and extra-clinical settings (Ewers et al., 2012). The acquisition of ESBL-genes happened over time, and the initial spread of ST131 most likely evolved from an emergence of chromosomally encoded fluoroquinolone resistance (Nicolas-Chanoine et al., 2014). Besides ST131, several STs, including ST648, ST617, ST167, ST410, ST224, and ST117, appear to be associated with ESBL-production, which demonstrates that ESBL-producing isolates are not equally distributed over all phylogenetic backgrounds (Ewers et al., 2012). This is expected in cases of solely plasmid-driven spread (Ewers et al., 2012). Regarding the success of pathogenic clonal lineages of B2-ST131, similar scenarios might also apply to CTX-M-producing lineages of ST648 belonging to phylogenetic group D, also known for harboring virulent isolates (Pitout, 2012; Ewers et al., 2014). Many strains of ST131 and ST648 carry plasmids encoding ESBL-enzymes, often of the CTX-M-15 type, and have become problematic due to limitations in antimicrobial therapies (Johnson et al., 2010). Besides ESBL-encoding genes, plasmids of these *E. coli* isolates contain antibiotic resistance determinants affecting various antimicrobial classes, which often results in multi-drug resistant phenotypes (Woodford et al., 2011). Prior studies suggest trade-offs between antibiotic-resistance and fitness in such strains (Dasilva and Bailey, 1986; Lenski, 1997; Andersson and Hughes, 2010). However, this does not necessarily apply to ST131 and ST648. In contrast, the combination of multi-resistance, virulence and phylogenetic background is hypothesized to be a recipe for their successful pandemic spread (Johnson et al., 2010; Pitout, 2012; Calhau et al., 2013). In addition to antibiotic resistance genes, ESBL-plasmids harbor non-resistance factors, which are partly unexplored. These include fertility and virulence factors, genes for plasmid maintenance including toxin-antitoxin systems, resistances against heavy metals (Seiler and Berendonk, 2012; Schaufler et al., 2013), and putative protein-coding genes (Smet et al., 2010b).

Why do ESBL-associated STs exist, and why are certain clonal lineages so successful not only in environments with high and moderate antibiotic pressures, but also in antimicrobially isolated areas? It might be due to their ubiquity and frequent detection, whereas rare lineages are found less often, or for reasons beyond antibiotic resistance such as virulence-associated factors. One possibility has rarely been studied: the interaction between ESBL-plasmids and the chromosomal content of particular clonal lineages. The influence of non-resistance genes on the chromosome may be of particular importance. For the closely related species *Klebsiella pneumoniae*, it was shown that acquisition of ESBL-plasmids lead to expression changes of chromosomally encoded fimbriae genes, subsequently affecting the overall invasion ability of tested strains (Sahly et al., 2008). Another main bacterial virulence factor is the chromosomally encoded ability to form biofilms. The subtle interactions between biofilm formation and its counterpart, motility, are mostly regulated by the transcriptional regulator *csgD* (curlin subunit gene D; Hammar et al., 1995; Dudin et al., 2014). Biofilm formation has previously been linked to antimicrobial resistance (Ito et al., 2009); however, the influence of ESBL-plasmids on *csgD*-associated virulence features remains to be investigated.

This study addressed two hypotheses: (i) ESBL-plasmid carriage does not negatively influence the host's growth/metabolic fitness; and (ii) ESBL-plasmid carriage supports the host through the ESBL-plasmid's influence on chromosomally encoded virulence-associated features.

MATERIALS AND METHODS

Strains

Seven wild-type (WT) ESBL-producing *E. coli* strains, their ESBL-"plasmid" cured variants (PCV) (Schaufler et al., 2013), and transformants (T) with the reintroduced large ESBL-plasmid constructed from PCVs (Green and Sambrook, 2012) were analyzed in this study; in total, 21 strains were studied. The WT B2-ST131 and D-ST648 strains originated from different hosts including humans, companion animals, and wild birds (Supplementary Table S1).

ESBL-Plasmid-"Curing" and Transformations

As previously described by Schaufler et al. (2013), large ESBL-plasmids were extracted from seven WT ESBL-carrying *E. coli* strains using a heat technique (Dale and Park, 2004). Loss of the large ESBL-plasmid as well as the clonal character of WT and its corresponding PCV strain were tested via plasmid-profile analysis and *Xba*I-pulsed-field gel electrophoresis (PFGE) (Schierack et al., 2009). To assure genetic identity and to exclude chromosomal changes in the PCV, the whole genome sequences of WT and PCV strains were analyzed using bioinformatics. This included a pairwise comparison of the number of orthologous genes using the OrthoMCL pipeline (Chen et al., 2006) and computation of the phylogenetic distances of all strains based on their Maximum Common Genome (MCG) (Von Mentzer et al., 2014) as previously described (Schaufler et al., 2013). Transformants with the reintroduced large ESBL-plasmid were constructed to verify the observed phenotypic differences (Supplementary Table S1). PCVs functioned as electrocompetent acceptor strains; thus, the respective large ESBL-plasmid was transformed via electroporation (Green and Sambrook, 2012). Transformed

strains were screened for ESBL-production on cefotaxime (4 µg/ mL; Sigma-Aldrich, Taufkirchen, Germany) containing CHROMagar™ (MAST Diagnostica, Reinfeld, Germany) plates for ESBL-enzyme-production. Plasmid profile analysis was used to evaluate the success of electroporation.

ESBL-Plasmid Characteristics

Whole genomes of all WT and their corresponding PCV strains were sequenced using an Illumina HiSeq2000 sequencer in collaboration with the Wellcome Trust Sanger Institute (Cambridge, United Kingdom). The resulting reads were used for a *de novo* assembly (CLC Genomics Workbench 6.5, CLC Bio, Denmark). PLACNET analysis (Lanza et al., 2014) was then performed for all genomes to extract putative plasmid contiguous sequences (contigs) and to assign them to the large ESBL-plasmid and to smaller, additional non-ESBL-plasmids. These were confirmed via BLAST analysis, in which they were compared to reference sequences of known plasmids. All previously defined plasmid contigs showed a high similarity to parts of plasmids that have already been described. Annotation of the contigs was performed using the annotation feature of the program Geneious version 7.1.2 (Kearse et al., 2012) with 100% similarity to an in-house plasmid reference data base (data not shown). ESBL types, relaxase (REL) and plasmid replication initiator (RIP) proteins, incompatibility (Inc) groups, approximate ESBL-plasmid sizes, the number of additional plasmids, and virulence factors were detected based on PLACNET ESBL-plasmid sequences and using VirulenceFinder 1.4 (Joensen et al., 2014) and ResFinder 2.1 (Zankari et al., 2012; **Table 1**). BLAST ring image generator (BRIG) (Alikhan et al., 2011) was used to visualize the annotated PLACNET ESBL-plasmid sequences (pIMT17433, pIMT19205, pIMT27685, pIMT16316, pIMT17887, pIMT21183, and pIMT23463) of the seven WT strains with pEC_L8 as a reference ESBL-plasmid (Smet et al., 2010b; **Figure 1**).

Growth Curves

Growth curves in LB medium were performed in triplicate using standard protocols.

Omnilog® Phenotype Microarray (PM)

Using 96-well microtiter plates spotted with different substrates, growth/metabolic activity in 379 single substrates and sensitivity to 48 antimicrobial and chemical compounds was analyzed for all strains [Omnilog® (Biolog, Hayward, USA), Supplementary Table S2]. Three biological replicates were tested on six PM plates (PM1 and PM2: carbon sources; PM3: nitrogen sources; PM4: phosphorus and sulfur sources [http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf]; PM13 and PM14: chemical sensitivity [http://www.biolog.com/pdf/pm_lit/PM11-PM20.pdf]). Plates were inoculated with the bacterial suspension according to Omnilog® PM protocols. Plates were incubated for 48 h at 37°C. Respiration was measured by dye (tetrazolium violet) reduction every 15 min. The calculated longitudinal respiration kinetics were analyzed in R (Vaas et al., 2013). In confidence interval plots computed with extract defined by enlisted metadata for the parameter "area under the curve," only wells in which the normalized mean point estimates of WT and T groups showed

TABLE 1 | Characteristics of large ESBL-plasmids of the seven ESBL-producing wild-type strains.

Strain	ESBL type	ESBL-plasmid REL/RIP proteins and Inc group	ESBL-plasmid size (bp)	Number of additional plasmids (sizes in bp)	ESBL-plasmid additional resistance genes	ESBL-plasmid virulence genes
IMT17433	CTX-M-15	MOB_{F12}, RepFIA/FII, IncF	122,823	1 (1600)	bla_{TEM-1}, bla_{OXA-1}, *tet(A), tet(R), aadA, aac(6')-Ib-cr, catB4*	*finO, traT*
IMT19205	CTX-M-27	MOB_{F12}, RepFIA/FIB/FII, IncF	166,151	2 (1500, 5200)	bla_{TEM-1}, *tet(A), sul2, strA, aadA, aac(3)-IId, aac(3)-IV, aac(6')-Ib-cr*	*finO, traT, senB*
IMT27685	CTX-M-15	MOB_{F12}, RepFIA/FII, IncF	133,611	1 (1600)	bla_{OXA-1}, *tet(A), sul1, strA, strB, aadA, aac(6')-Ib-cr, dfrA17*	*traT*
IMT16316	CTX-M-15	MOB_{F12}, RepFIA/FIB, IncF	136,508	4 (2500, 3200, 4100, 7000)	bla_{TEM-1}, *tet(A), tet(R), sul1, sul2, strA, strB, aadA, aac(3)-II, dhfrVII, dfrA17*	*finO, traT*
IMT17887	CTX-M-15	MOB_{F12}, RepFIA/FIB, IncF	143,494	2 (2600, 7100)	bla_{TEM-1}, *tet(A), tet(R), sul1, sul2, strA, strB, aadA, aac(3)-IId, aph(3')-Ia, dhfrVII, dfrA17*	*finO, traT*
IMT21183	CTX-M-15	delta-TraI, RepFIA, IncF	103,420	1 (7100)	bla_{TEM-1}, *tet(A), tet(R), sul1, sul2, strA, strB, aadA, aac(3)-II, aph(3')-Ia, dhfrVII, dfrA17*	*traT*
IMT23463	CTX-M-14	MOB_{F12}, RepFIB/FII, IncF	143,193	1 (4100)	bla_{TEM-1}, bla_{OXA-1}, *tet(A), sul2, strA, strB, aadA, aac(3)-IId, aac(6')-Ib-cr, dfrA17*	*finO, traT, sitABCD, cma*

REL, relaxase; RIP, replication initiator protein; Inc, incompatibility; bp, base pairs.

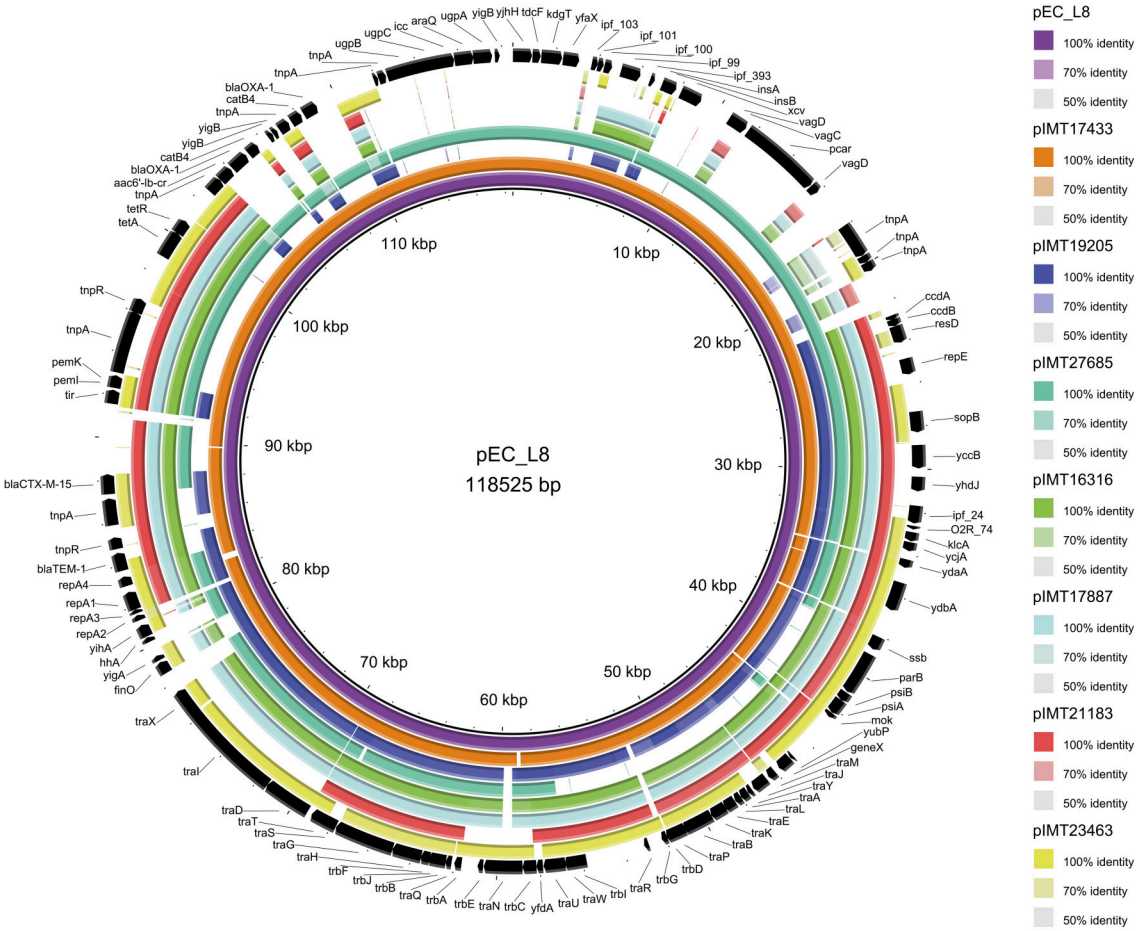

FIGURE 1 | Circular visualization of the seven wild-type ESBL-plasmid sequences as compared to *E. coli* ESBL-plasmid pEC_L8 using BRIG (Alikhan et al., 2011).

no overlapping 95% confidence intervals as compared to those of the PCV group, were considered to be significantly different. The group means of these wells underwent further statistical analysis using Tukey's method for multiple comparisons.

Macrocolonies

Three microliters of overnight culture (from a single colony grown in 5 mL BHI broth) from all strains were dropped on span agar plates (H. Carroux, Germany) with or without sodium chloride (5%) and congo red solution [0.5% congo red (Sigma-Aldrich, Taufkirchen, Germany) and 0.25% coomassie-brilliant-blue (Carl Roth, Karlsruhe, Germany) diluted in ethanol]. Plates were incubated for 5 days at 28°C (Romling, 2005; Richter, 2011). Following an initial comprehensive screening to detect differences between WT and PCV strains regarding their cellulose and/or curli fimbriae production, follow-up runs were performed on plates containing and lacking sodium chloride for all strains (**Table 2**). These follow-up runs were repeated six times. Reference strains (AAEC189, Blomfield et al., 1991, IMT26949, and W3110 Hayashi et al., 2006) were included in all runs for all plates and at all temperatures.

TABLE 2 | Cellulose and curli fimbriae expression by 17433 and 16316 strains on span agar plates with congo red, with or without sodium chloride, incubated at 28°C for 5 days.

Strain	With sodium chloride		Without sodium chloride	
	Cellulose	Curli	Cellulose	Curli
IMT17433	******	******	******	******
PCV17433				
T17433	*****	******	*****	******
IMT16316	******	******		
PCV16316		******		
T16316	******	******		

*Six repetition runs followed a comprehensive initial screening. *, positive cellulose and/or curli fimbriae phenotype per run; 16316 strains were not tested on plates lacking sodium chloride as they did not show any differences during the initial screening.*

Biofilm Formation Assays

Overnight culture (from a single colony grown in 5 mL LB and minimal medium M63 overnight, Pardee et al., 1959) was set to optical density $OD_{600} = 0.05$. Technical triplicates of

the suspension were added to a 96-well plate, which was then hermetically closed and incubated for 24 or 48 h at 28, 37, or 42°C. Reference strains (AAEC189 and W3110) were included in all runs in triplicate for each medium, at all temperatures and time points. After incubation, OD was measured with an ELISA-reader (Synergy HT, BioTEK Instruments, Bad Friedrichshall, Germany) and bacteria were washed with aqua bidestillata, fixed in 99% methanol, stained with 0.1% crystal violet, and dissolved in 80:20 ethanol:acetone. After dissolution, OD was again measured, then biofilm formation capacity was computed (Martinez-Medina et al., 2009) and statistically analyzed using IBM SPSS Statistics for Windows, Version 20 (Dunn, 2013). Normal distributions of measuring points of all groups (strains) were tested using Kolmogorov-Smirnov (Smirnov, 1948). Based on the non-normal distributions of the measuring points of all groups, the non-parametric Wilcoxon rank-sum test (Mann-Whitney *U*-test; Wilcoxon, 1945) was used to estimate whether the observed biofilm formation capacity differences were statistically significant ($p = 0.05$). This assay was repeated three times in triplicate per temperature, and in two media for all strains.

Motility Assays

Overnight culture (from a single colony grown in 5 mL BHI broth) was set to $OD_{600} = 1$. One milliliter was centrifuged and washed twice with 1 × phosphate buffered saline (PBS). Five microliters of the suspension were dropped onto swim-agar plates (LB and 0.3% agar). Plates were incubated at 28, 37, and 42°C. The strain MG1655 (Hayashi et al., 2006; Richter, 2011) was used as a control for a positive swimming phenotype. After 48 h, colony diameters were measured (Harshey, 2003) and statistically analyzed using IBM SPSS Statistics for Windows, Version 20 (Dunn, 2013). Normal distributions of measuring points of all groups (strains) were tested using Kolmogorov-Smirnov (Smirnov, 1948). Based on the non-normal distributions of the measuring points of all groups, the non-parametric Wilcoxon rank-sum test (Wilcoxon, 1945) was used to estimate whether the observed swimming differences were statistically significant. The significance level for multiple comparisons between WT, PCV and T groups was adjusted to $p = 0.016$. This assay was repeated six times.

RNA Sequencing

The RNA of IMT17433, PCV17433, and T17433 was sequenced. RNA was isolated from two biological replicates from each of two macrocolony and two motility plates. RNA was isolated from cells using the RNASnap method (Stead et al., 2012) and shipped to LGC Genomics (Berlin, Germany) for RNA sequencing with an Illumina HiSeq2000 producing one channel paired end reads. The details of the company's standard protocols for quality control, RNA extraction from the RNASnap technique and rRNA depletion (using Ribo-Zero (Epicentre), Biozym, Hessisch Oldendorf, Germany) can be found on their website (http://www.lgcgroup.com/services). cDNA synthesis, library generation, indexing and cluster generation were performed using Illumina technology (TruSeq RNA Sample Preparation Kit v2). Bioinformatic mRNA differential expression analysis included the following processing steps: (a) generating FastQC reports to check the quality of sequenced reads; (b) clipping Illumina TruSeq sequencing adapters from the 3′ ends of reads; (c) filtering rRNA reads using riboPicker (Schmieder et al., 2012); (d) aligning reads against IMT17433 as a reference using TopHat2 (Kim et al., 2013) and RSEM; (e) counting reads per gene using HTSeq-count, and per transcript and gene using RSEM (Li and Dewey, 2011); and (f) computing differential gene and transcript expression between different groups of samples (including technical and biological replicates) using R/Bioconductor packages DESeq (Anders and Huber, 2010), edgeR (Robinson et al., 2010), and Cuffdiff (part of the Cufflinks software package, Trapnell et al., 2013).

RESULTS

ESBL-Plasmid-"Curing" and Transformations

All PCVs kept their smaller plasmids and lost only the large ESBL-plasmid. Bioinformatic analysis of all WT and PCV genomes assured their genetic similarity, ruling out any changes in the PCVs' chromosomal content during the "curing" procedure (Schaufler et al., 2013; Von Mentzer et al., 2014). To verify detected phenotypic differences between WT and PCV strains, seven transformants containing the reintroduced large ESBL-plasmid were used (T17433, T19205, T27685, T16316, T17887, T21183, and T23463; Supplementary Table S1). These strains showed phenotypic cefotaxime-resistance (CLSI, 2008). Plasmid-profile analysis confirmed transformation of the large ESBL-plasmid.

ESBL-Plasmid Characteristics

Whole genomes of all WT strains were used for plasmid characterization (http://www.sanger.ac.uk/resources/downloads bacteria/escherichia-coli.html#project_2119; IMT17433 [ERR163891], IMT19205 [ERR163889], IMT16316 [ERR163879], IMT17887 [ERR163883], IMT21183 [ERR163880], IMT23463 [ERR163881]; http://www.sanger.ac.uk/resources/downloads/bacteria/escherichia-coli.html#project 2433; IMT27685 [ERR264283]). **Table 1** summarizes the most important ESBL-plasmid characteristics. Their sizes ranged from approximately 100–166 kb. Besides common bla_{CTX-M} types (mostly the CTX-M-15 enzyme), non-beta-lactam resistance genes (e.g., *tet(A)/(R)* and *aac(6′)-Ib-cr*) were found. Virulence-associated genes, mainly *finO* and *traT,* were present. All WT strains harbored at least one smaller non-ESBL-plasmid (**Table 1**). Analysis of PCV genomes showed complete loss of the MOB_{F12}/IncF ESBL-plasmids in six of the seven genomes analyzed. Strain IMT19205 contained two IncF plasmids that were unresolvable by PLACNET. This strain lost its ESBL-phenotype along with one of the IncF plasmids.

Figure 1 displays the annotated ESBL-plasmid sequences (pIMT17433, pIMT19205, pIMT27685, pIMT16316, pIMT17887, pIMT21183, and pIMT23463) as compared to ESBL-plasmid pEC_L8 (Smet et al., 2010a), showing both beta-lactam and non-beta-lactam resistance genes, as well as non-resistance genes, including *icc* (phosphodiesterase) and *hha*

(hemolysin expression modulating protein), which were present on all plasmids. Additionally, this comparison displays typical features of conjugative plasmids (*tra* regions and insertions sites) and plasmid partitioning (toxin-antitoxin systems: e.g., *vagC/D* and *pemI/K*).

In summary, although they contained similar genetic backbones and resistance determinants, ESBL-plasmids were rather diverse.

Growth Curves and Omnilog® Phenotype Microarray (PM)

No differences were detected between the LB growth curves of WT ESBL-producing *E. coli* strains and the corresponding PCVs (data not shown).

Growth/metabolic activity and chemical sensitivity of all strains were then screened using the Omnilog® PM system. Only a minor proportion of strains showed significant differences between the 427 tested compounds (Supplementary Table S2). Exemplary for plate PM1, significant differences were observed for strain combinations 17433 and 17887. Additionally, plates PM2 and PM3 revealed few significant differences (Supplementary Table S2). In contrast, on plate PM4 (sulfur and phosphorus sources), significant differences were observed for 22 wells, of which 18 showed higher values in WT and T strains, while four showed higher values in PCV strains.

The chemical sensitivity plates PM13 and PM14 revealed expected significant differences for ESBL-carrying (WT and T) strains in antimicrobial-containing wells, which showed higher metabolic values than corresponding PCV strains (e.g., PM13, C6: Doxycycline and D4: Cefuroxime; and PM14, G8: Carbenicillin; Supplementary Table S2).

In summary, given the high numbers of substrates tested, only a small proportion of wells showed significant differences in the "area under the curve" parameter between WT, PCV, and T strains. The detected significant differences were bidirectional, meaning that in some cases the PCV strains showed higher growth/metabolic activity values, and in other cases the ESBL-plasmid-carrying strains showed higher growth/metabolic activity values.

Macrocolonies

The expression of the biofilm-associated extracellular matrix components cellulose and/or curli fimbriae was tested using macrocolony assays. **Table 2** shows the results of the six follow-up runs, which were performed for those strains with differences in cellulose and/or curli fimbriae production during the initial screening (combinations 17433 and 16316).

On plates containing sodium chloride, ESBL-carrying WT IMT17433 expressed cellulose and curli fimbriae, whereas its corresponding PCV did not (**Figure 2**). Another mutant (PCV16316) did not show any cellulose expression in any run as compared to its associated WT strain (IMT16316), which produced both cellulose and curli fimbriae. In both cases, in accordance with the corresponding WT strains, the transformants T17433 (**Figure 2**) and T16316 produced curli fimbriae and cellulose in most runs. Only combination 17433 showed the same results on plates lacking sodium chloride (**Table 2**).

In summary, PCV17433 and PCV16316 displayed reduced production of extracellular components as compared to their respective wild-type and transformant strains.

Biofilm Formation Assays

Virulence-associated biofilm assays revealed significant differences among three WT/PCV combinations (IMT/PCV17433, IMT/PCV27685, and IMT/PCV17887) after 24 h and among two combinations (IMT/PCV17433 and IMT/PCV17887) after 48 h. IMT17433 and its corresponding PCV were particularly interesting. At all three temperatures and after both time points, the WT strain showed an enhanced biofilm formation capacity in glucose-containing M63 medium (28°C, 24 h: $p = 0.222$; 37°C, 24 h: $p = 0.008$; 42°C, 24 h: $p = 0.094$; 28°C, 48 h: $p < 0.001$; 37°C, 48 h: $p < 0.001$; 42°C, 48 h: $p = 0.008$) as compared to the PCV strain. In contrast, biofilm formation by PCV17433 was better in LB medium

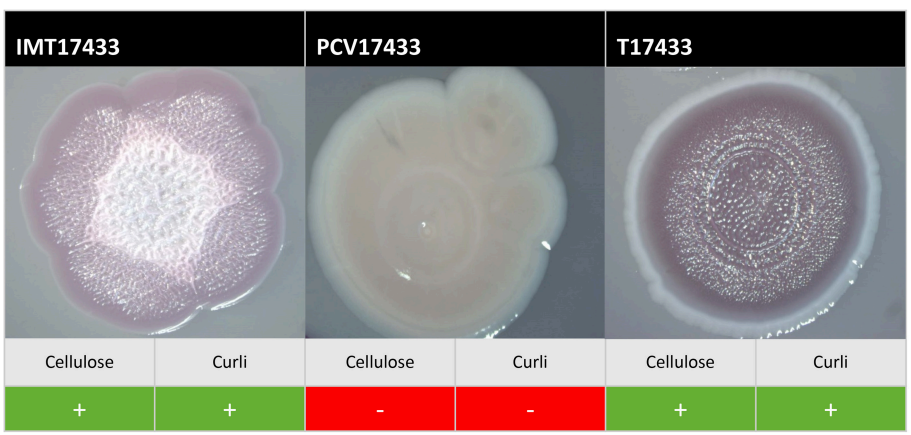

FIGURE 2 | Exemplary macrocolonies of IMT/PCV/T17433. Span agar plates with congo red and sodium chloride, incubated for 5 days at 28°C.

(28°C, 24 h: $p < 0.001$; 37°C, 24 h: $p < 0.001$; 42°C, 24 h: $p < 0.001$; 28°C, 48 h: $p = 0.006$; 37°C, 48 h: $p = 0.004$; 42°C, 48 h: $p = 0.002$; **Figures 3A,B**). Transformants did not show any biofilm formation.

In summary, some WT and PCV combinations showed differences in their biofilm formation capacities in both directions; these results were medium-dependent.

Motility Assays

Motility assays were performed to test the swimming capacity of the strains at different temperatures. Two PCV strains (PCV17433 and PCV17887) showed significantly increased swimming capacity as compared to their corresponding WT strains (IMT17433 and IMT17887) at 28, 37, and 42°C (IMT/PCV17433: 28°C, $p = 0.002$; 37°C, $p = 0.002$; 42°C, $p = 0.006$; IMT/PCV17887: 28°C, $p = 0.002$; 37°C, $p = 0.002$; 42°C, $p = 0.004$). Both transformants (T17433 and T17887) showed significantly reduced swimming capacity as compared to their corresponding PCV strains, with the exception of T17887 at 28°C. (T/PCV17433: 28°C, $p = 0.002$; 37°C, $p = 0.002$; 42°C, $p = 0.002$; T/PCV17887: 28°C, $p = 0.032$; 37°C, $p = 0.004$; 42°C, $p = 0.013$; **Figures 4**, **5A–C**).

In summary, PCV17433 and PCV17887 showed higher swimming capacity than their respective WT and T strains.

RNA Sequencing

To gain insight into differential gene expression, RNA sequencing was performed for IMT/PCV/T17433. Only up- or downregulated genes detected with all three software packages and a threshold of 1.5 fold bidirectional regulation were considered for subsequent analysis.

Cellulose- and curli fimbriae-related differentially regulated genes from the macrocolony assay included: *csgB* (upregulation in WT compared to PCV: 9.8), *csgA* (8.6), *csgE* (6.0), *csgF* (5.5), *csgD* (5.5), *csgG* (4.0), *csgC* (3.6), and *adrA* (2.3). Differentially regulated genes important for swimming in the motility assay were, among others: *fliZ* (downregulation in WT compared to PCV: −1.9), *flgH* (−2.0), *flgF* (−2.5), *flhC* (−2.7), *fliD* (−2.7), *flgL* (−3.0), *flgK* (−3.1), *flgC* (−3.4), *flhD* (−3.5), *fliL* (−4.0), *flgD* (−4.2), and *fliC* (−5.7). Most genes were found to be up- or downregulated in both assays, except for *csgD* and *csgC*, which were only found to be upregulated in the macrocolony assay in the WT strain as compared to the PCV strain. Conversely, downregulation of *flhC* and *flhD* was only observed in the motility assay. All cellulose- and curli-related genes that were upregulated in the macrocolony assay in IMT17433 as compared to PCV17433, were also upregulated in T17433 as compared to PCV17433. Swimming-related genes that were downregulated in the motility assay were not detected in the transformant (e.g., *fliZ, flgH, flgA, flgL, flgG, fliC*).

In summary, RNA sequencing verified the observed phenotypes for WT17433, PCV17433, and T17433 at the transcriptional level.

Candidate Genes

The observed phenotypic and transcriptomic differences were only explainable by differences at the genetic level. With only the ESBL-plasmids differing between WT/T and PCV strains and resistance determinants not explaining the results, the next step in this study was to focus on the non-resistance genes encoded by these plasmids. **Table 3** shows candidate genes of the seven ESBL-plasmid sequences based on PLACNET analysis. Previous studies suggest their involvement in biofilm formation and motility. The candidate genes included *hha* (encoding the hemolysin expression modulating protein) and *yihA* (encoding a cell division protein), which were both present on pIMT17433, pIMT19205, and pIMT27685. The genes *icc* (encoding a phosphodiesterase) and *yfaX* (encoding a putative transcriptional factor) were encoded on pIMT17433 and pIMT27685. All ESBL-plasmids carried *tra* genes important for conjugation.

In summary, the detected candidate genes were mostly encoded on ESBL-plasmids of sequence type ST131 (pIMT17433, pIMT19205, and pIMT27685).

DISCUSSION

The success of ESBL-producing *E. coli*, particularly the pandemic pathogenic clonal lineages, cannot be explained by antimicrobial resistance alone. To assess the possibility of interactions between ESBL-plasmids and chromosomal content, we conducted a study using an unconventional approach by constructing PCVs of ESBL-plasmid-carrying WT strains of ST131 and ST648. Based on growth curves in LB medium and detailed growth/metabolic activity under different conditions, WT and PCV strains showed similar behaviors and were thus regarded suitable for analysis in subsequent phenotypic assays. Phenotypic differences between WT and PCV strains were generally verified by T strains.

In PM assays, which are a consolidation of regular LB growth curve tests (Supplementary Table S2), significant differences were detected in select wells (plates PM1-4: 3% among all wells without combination 17433). When the results of PM4 were included for 17433, 15% of the wells showed differences; however, these results need to be treated with caution since the negative control (A01) had high values for PCV17433 even after several repetitions of the assay. High values for negative controls in PM assays have previously been described for *E. coli* (Vaas et al., 2012). Differences in antimicrobial sensitivity assays on plates PM13 and PM14 between ESBL-plasmid-carrying strains and PCVs are explainable by loss of ESBL-plasmids in PCVs, which, besides ESBL-genes, carry additional antimicrobial resistance genes (**Table 1**; e.g., PM13, C6: Doxycycline and D4: Cefuroxime; and PM14, G8: Carbenicillin). Overall, significant differences were bidirectional, meaning that each of the ESBL-plasmid-carrying strains and the PCVs showed higher values in select wells. This indicates, as underscored by LB growth curve results, that ESBL-plasmid-carriage does not necessarily lead to fitness loss. This contradicts the hypothesis that considerable trade-offs exist between antimicrobial resistance and fitness (Dasilva and Bailey, 1986; Lenski, 1997; Andersson and Hughes, 2010). Our finding applied to all seven strain combinations irrespective of origin, ST, or ESBL-plasmid characteristics.

Biofilm-related macrocolonies result from bacterial incubation over several days, where the biosynthesis of the

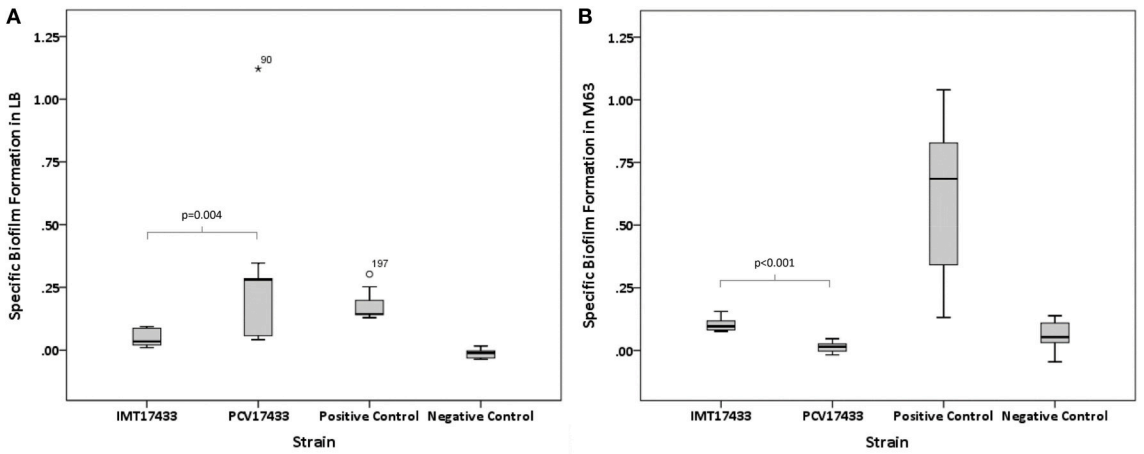

FIGURE 3 | (A) Box plots of the distributions of the specific biofilm formation capacities of IMT17433 and PCV17433 at 37°C after 48 h in LB medium. Plots were generated using IBM SPSS Statistics for Windows, Version 20. Positive control, AAEC189; Negative control, W3110. **(B)** Box plots of the distributions of the specific biofilm formation capacities of IMT17433 and PCV17433 at 37°C after 48 h in M63 medium. Plots were generated using IBM SPSS Statistics for Windows, Version 20. Positive control, AAEC189; Negative control, W3110.

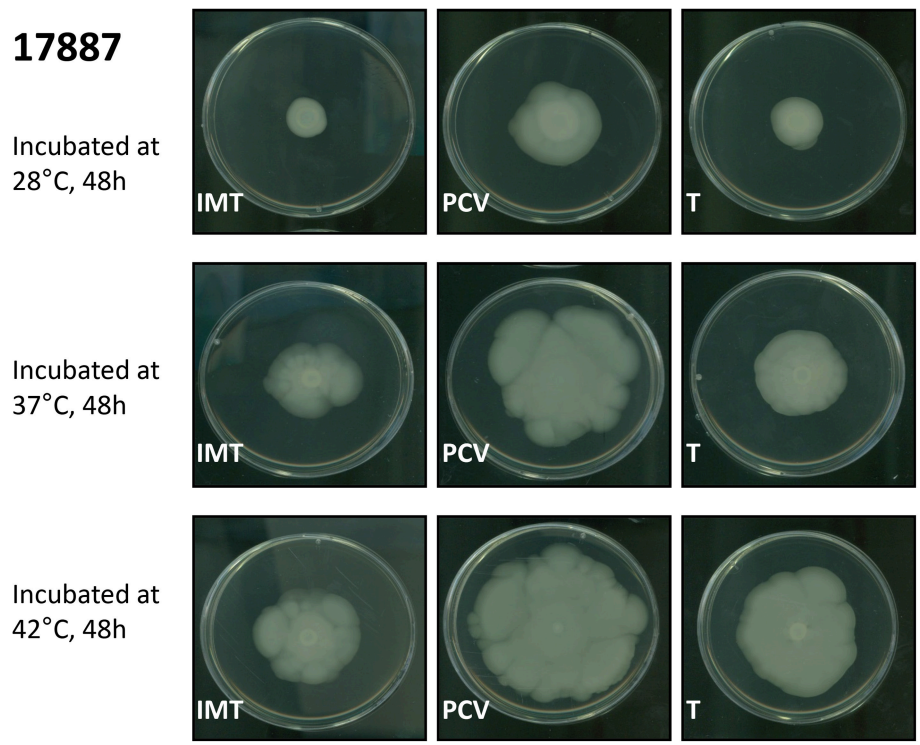

FIGURE 4 | Exemplary swimming motility of IMT/PCV/T17887. LB plates wih 0.3% agar. Incubation at 28, 37, and 42°C for 48 h.

important virulence-related extracellular matrix components cellulose and curli fimbriae typically occurs below 30°C (Bokranz et al., 2005; Richter et al., 2014). Curli fimbriae promote adhesions to abiotic surfaces (Zogaj et al., 2001) and are associated with virulence, as they play key roles during internalization into epithelial cells (Gophna et al., 2001) and persistence in avian guts (La Ragione et al., 1999). Bacteria produce cellulose mainly as protection from both chemical and mechanical influences (Ross et al., 1991). Considering the results of the macrocolony assays for those combinations with differences, the enhanced ability of ESBL-plasmid-carrying WT and T strains to synthesize curli fimbriae and/or cellulose

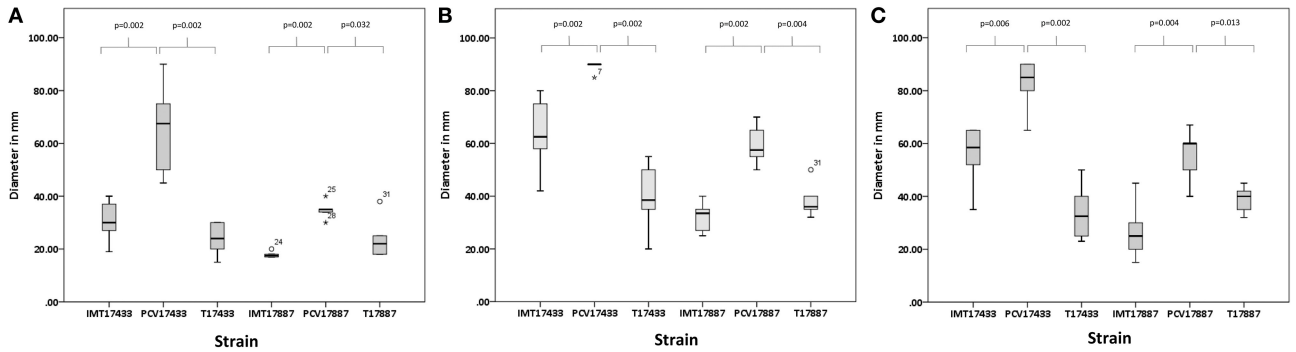

FIGURE 5 | (A) Box plots of the distributions of the diameters, in millimetres, of the swimming capacities of IMT/PCV/T17433 and IMT/PCV/T17887 at 28°C. Plots were generated using IBM SPSS Statistics for Windows, Version 20. **(B)** Box plots of the distributions of the diameters, in millimetres, of the swimming capacities of IMT/PCV/T17433 and IMT/PCV/T17887 at 37°C. Plots were generated using IBM SPSS Statistics for Windows, Version 20. **(C)** Box plots of the distributions of the diameters, in millimetres, of the swimming capacities of IMT/PCV/T17433 and IMT/PCV/T17887 at 42°C. Plots were generated using IBM SPSS Statistics for Windows, Version 20.

TABLE 3 | Candidate genes based on annotated PLACNET ESBL-plasmid sequences, literature survey, and RNA sequencing results.

Gene	Protein	Predicted function	Presence	References
hha	Hemolysin expression modulating protein	Involved in biofilm formation and motility	pIMT17433, pIMT19205, pIMT27685	Barrios et al., 2006
icc	Phosphodiesterase	Involved in biofilm formation	pIMT17433, pIMT27685	Kalivoda et al., 2013
yfaX	Predicted DNA-binding transcriptional regulator	Putative HTH-type transcription factor	pIMT17433, pIMT27685	Perez-Rueda and Collado-Vides, 2000
yihA	Cell division protein, predicted checkpoint GTPase	GTP binding	pIMT17433, pIMT19205, pIMT27685	Lehoux et al., 2003
tra	Transfer regions of the F-conjugative plasmid	Involved in biofilm formation	all seven pIMT ESBL-plasmids	Ghigo, 2001

in contrast to their respective PCV strains was particularly notable. This indicates that ESBL-plasmid carriage confers benefits in terms of biosynthesis of virulent- and survival-associated extracellular matrix components to some bacterial strains. Several prior studies have described an influence of conjugative plasmids on biofilm formation (Ghigo, 2001; Yang et al., 2008). May and Okabe (May and Okabe, 2008) investigated the influence of natural IncF F-plasmids on biofilm formation and maturation. In this study, both conjugative (*tra* regions) and non-conjugative plasmid genes seemed to play a role. In our study, all seven ESBL-plasmids belonged to incompatibility group IncF. Furthermore, all encoded different *tra* genes are important for conjugation; however, since not all WT strains showed differences in extracellular matrix component production as compared to PCV strains, these factors are probably not solely responsible for our observed results.

Biofilm results from IMT/PCV17433 underline the complexity of biofilm formation, which is also dependent on nutrient availability. The enhanced ability of IMT17433 to form biofilms in M63 medium as compared to PCV17433 may point toward plasmid encoded features that enable the WT strain to use limited nutrients efficiently, perhaps via phosphate-dependent pathways. PM results revealing that select phosphate-containing wells in which IMT17433 showed higher respiration values than PCV17433 reinforce the latter hypothesis. Explanations of the observed enhanced ability of the PCV strain to form biofilms in LB medium, however, remain speculative. There may have been no need for planktonic IMT17433 to transform into a biofilm due to optimal utilization of the rich LB medium. A switch from a sessile (multicellular behavior, biofilm) to a planktonic (motility) way of life, and vice versa, underlies subtle interactions at a molecular level that include numerous complex cascades. Flagellar biosynthesis, for instance, seems not only crucial for bacterial swimming but also leads to surface colonization and subsequent biofilm formation. Insufficient nutrient supply leads to detachment of the cells from the biofilm and adoption of a planktonic lifestyle (Harshey, 2003).

Explanations of the observed enhanced swimming capacity among some PCV strains as compared to their corresponding WT and T strains in the motility assays remain similarly speculative. Flagellar synthesis has been shown to be energetically costly, and ESBL-plasmid-carrying strains might use their energy more strategically (Zhao et al., 2007). Alternately, due to better nutrient utilization, ESBL-plasmid-carrying strains may not have to swim as well as PCV strains to reach peripheral zones with, presumably, richer nutrition supplies.

Phenotypic differences in cellulose and curli fimbriae production as well as swimming capacity, particularly those

observed between the 17433 strains, were confirmed by RNA sequencing results showing upregulation of chromosomally encoded extracellular matrix-related genes and downregulation of chromosomally encoded flagellar-related genes in IMT17433 as compared to PCV17433. Reversible RNA sequencing results for T17433 strengthen the reliability of the differences seen at phenotypic and transcriptomic levels. The key role of the transcription factor *csgD* is notable in biofilm formation and motility capacity. *csgD* regulates not only the second curli fimbriae operon *csgBAC* and *adrA,* whose product is not only accountable for several other genes involved in cellulose production, but also influences *flhDC*, the main operon for flagellar synthesis and thus motility (Ogasawara et al., 2011; Chambers and Sauer, 2013). In our study, RNA sequencing results revealed upregulation of *csgD, csgBAC,* and *adrA* and downregulation of *flhDC* in IMT17433 as compared to PCV17433. These results not only confirm the phenotypic data, but also emphasize the central importance of the CsgD protein in regulating the subtle interaction between bacterial sessile and planktonic ways of life.

Overall, the phenotypic tests performed for all seven strain combinations did not show a consistent pattern. Although, this is expected due to high diversity among the group of ESBL-plasmids as well as in the origins of host strains and their phylogenetic backgrounds, such inconsistencies weaken the generalizability of our conclusions. Rather, our results should be seen as an impetus for prospective studies. Interestingly, despite ESBL-plasmid diversity, each of pIMT17433, pIMT19205, and pIMT27685 carried different candidate genes, including *hha* and *icc*, which both have been described to be involved in biofilm formation and motility (Barrios et al., 2006; Kalivoda et al., 2013). Only further experiments that include cloning and knockout methods will provide more information on underlying molecular mechanisms.

In conclusion, this pilot study showed that ESBL-plasmid carriage does not necessarily lead to a growth/metabolic fitness loss. Some ESBL-plasmids in select strains may possess the potential to influence chromosomal gene expression, particularly of those genes that are important for the subtle interactions between the sessile and planktonic ways of life, such as *csgD*. We hypothesize that this may contribute to their virulence potential and pandemic success in different habitats, although underlying mechanisms remain to be identified and characterized.

AUTHOR CONTRIBUTIONS

All authors gave their final approval of the version to be published. Conception and Design: SG, CE, KS, LW, FD. Data analysis: KS, SG, TS, DP, MD. Data acquisition: KS, SG, TS. Writing: KS, SG, LW, CE, DP, TS, MD, FD.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00336

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Fitness Cost of Fluoroquinolone Resistance in Clinical Isolates of *Pseudomonas aeruginosa* Differs by Type III Secretion Genotype

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Fluoroquinolone (FQ) resistance is highly prevalent among clinical strains of *Pseudomonas aeruginosa*, limiting treatment options. We have reported previously that highly virulent strains containing the *exoU* gene of the type III secretion system are more likely to be FQ-resistant than strains containing the *exoS* gene, as well as more likely to acquire resistance-conferring mutations in *gyrA/B* and *parC/E*. We hypothesize that FQ-resistance imposes a lower fitness cost on *exoU* compared to *exoS* strains, thus allowing for better adaptation to the FQ-rich clinical environment. We created isogenic mutants containing a common FQ-resistance conferring point mutation in *parC* from three *exoU* to three *exoS* clinical isolates and tested fitness *in vitro* using head-to-head competition assays. The mutation differentially affected fitness in the *exoU* and *exoS* strains tested. While the addition of the *parC* mutation dramatically increased fitness in one of the *exoU* strains leaving the other two unaffected, all three *exoS* strains displayed a general decrease in fitness. In addition, we found that *exoU* strains may be able to compensate for the fitness costs associated with the mutation through better regulation of supercoiling compared to the *exoS* strains. These results may provide a biological explanation for the observed predominance of the virulent *exoU* genotype in FQ-resistant clinical subpopulations and represent the first investigation into potential differences in fitness costs of FQ-resistance that are linked to the virulence genotype of *P. aeruginosa*. Understanding the fitness costs of antibiotic resistance and possibilities of compensation for these costs is essential for the rational development of strategies to combat the problem of antibiotic resistance.

Keywords: ***Pseudomonas aeruginosa,*** **fluoroquinolone resistance, type III secretion, fitness, virulence**

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative pathogen that is the leading cause of nosocomial pneumonia (Restrepo and Anzueto, 2009; Quartin et al., 2013). Resistance to the fluoroquinolone (FQ) antibiotics has risen dramatically due to the widespread prescribing of this class of drug, limiting treatment options for *P. aeruginosa* infections (Linder et al., 2005). *P. aeruginosa* acquires resistance to the FQs through mutations in genes regulating the expression of efflux pumps and through point mutations in target site genes. The target enzymes of the FQs are the type II

topoisomerases, GyrA/B and ParC/E (Hooper, 2001). Resistance-conferring mutations in these genes, known as target site mutations, have been well described in *P. aeruginosa* (Nakano et al., 1997; Mouneimné et al., 1999; Higgins et al., 2003).

Pseudomonas aeruginosa has the ability to cause a variety of severe infections due to its many virulence factors. Specifically, *P. aeruginosa* utilizes the type III secretion system (TTSS) during acute infections to evade phagocytosis, invade host cells, and cause cell death (Veesenmeyer et al., 2009). The TTSS consists of a molecular syringe-like apparatus that extends through the inner and outer membranes and directly contacts the host cell. This allows effector toxins (ExoU, ExoS, ExoY, and/or ExoT) to be directly injected into the cytoplasm of host cells. Although residing at entirely separate loci, the genes encoding the toxins ExoU and ExoS are mutually exclusive in most strains, with the *exoS* genotype accounting for about 70% of clinical and environmental strains (Feltman et al., 2001; Garey et al., 2008). While less prevalent in general, *exoU* strains are more virulent than *exoS* strains, as has been shown in animal models of acute pneumonia (Shaver and Hauser, 2004). Importantly, infection with these strains leads to poor outcomes in patients with ventilator-associated pneumonia (Roy-Burman et al., 2001; El Solh et al., 2008) as well as increased persistence and severity of disease (Schulert et al., 2003).

Alarmingly, clinical studies have shown a correlation between FQ-resistance and virulence. We have previously reported that patients infected with FQ-resistant strains of *P. aeruginosa* had threefold higher mortality or prolonged illness by an additional 5 days compared to those infected with FQ-susceptible strains (Hsu et al., 2005). In addition, clinical isolates with the more virulent *exoU* genotype were shown to more likely be FQ-resistant than *exoS* strains (Wong-Beringer et al., 2008; Agnello and Wong-Beringer, 2012). Others have reported similar results in isolates from various infection sites (Zhang et al., 2014; Peña et al., 2015). Furthermore, in a separate study, we found that the combined traits of FQ-resistance and *exoU* genotype among respiratory isolates of *P. aeruginosa* were significantly associated with the development of pneumonia rather than bronchitis or colonization (Sullivan et al., 2014), suggesting that resistance and virulence traits may be linked, negatively impacting disease severity.

In a large study of 270 clinical isolates, we found that significantly more *exoU* strains were FQ-resistant, compared to *exoS* strains (63% vs. 49%, $p = 0.03$). Sequencing of the FQ target site genes *gyrA/B* and *parC/E* revealed that *exoU* strains were more likely than *exoS* strains to acquire two or more resistance-conferring target site mutations (Agnello and Wong-Beringer, 2012). Specifically, we found that while FQ-resistant *exoU* and *exoS* strains were similarly likely to possess a mutation in *gyrA*, *exoU* isolates were more likely to also have acquired an additional mutation in *parC*, resulting in greatly increased levels of resistance. While there is mounting evidence for the co-selection of resistance and virulence traits among pathogenic bacteria (Beceiro et al., 2013), the biological mechanisms underlying these observations are not well understood. Since the identification of the ExoU and ExoS toxins of the *P. aeruginosa* type III secretion system, many studies have investigated the roles of each toxin during infection (Allewelt et al., 2000; Schulert et al., 2003; Shaver and Hauser, 2004, 2006) while others have described the association of *exoU* strains with increased FQ resistance (Lakkis and Fleiszig, 2001; Maatallah et al., 2011) supporting our own observations. The aim of the current study was to gain insights into the biological basis underlying the differential development of resistance in *exoU* vs. *exoS* strains, which previous studies have not explored. We hypothesize that the greater propensity of *exoU* strains to acquire multiple target site mutations compared to *exoS* strains reflects a difference in the fitness costs associated with FQ-resistance mutations, favoring the strains with the *exoU* genetic background.

A recent meta-analysis of studies conducted on fitness costs of resistance to a variety of antibiotic classes in different organisms showed that most point mutations are generally costly (Melnyk et al., 2015). However, there is great variability depending on organism, drug, and mechanism of resistance. Furthermore, fitness costs can be mitigated through the accumulation of compensatory mutations that restore fitness to wild-type levels without loss of resistance (Andersson and Hughes, 2010). FQ-conferring mutations have been shown to impose variable fitness costs, depending on strain background and specific combinations of mutations (Komp Lindgren et al., 2005; Luo et al., 2005; Marcusson et al., 2009; Machuca et al., 2014; Wasels et al., 2015).

In the current study, we compared the fitness effects of a target site mutation in *parC* using isogenic mutants created from clinical *exoU* and *exoS* isolates. We investigated the fitness effects of this mutation using *in vitro* head-to-head competition experiments. Changes in supercoiling level and mutation frequency between mutants and parent strains were also assessed to explore potential mechanisms for the difference in fitness observed.

Our results suggest that the FQ resistance-conferring point mutation in *parC* studied here may confer less of a fitness cost to *exoU* strains than to *exoS* strains, with *exoU* strains showing evidence of compensation for the fitness costs. These results provide a potential biological explanation for the observed predominance of *exoU* strains in the clinical FQ-resistant population. The co-selection of FQ-resistance in highly virulent *exoU* strains will likely have a negative impact on patient outcomes, which underscores the importance to gain a better understanding of the biological basis for this observation.

MATERIALS AND METHODS

Ethics Statement

The institutional review boards at both Huntington Hospital and the University of Southern California have approved this study. Informed consent was waived from all participants since bacteria cultures were saved as part of a longitudinal epidemiological surveillance study. All data from respiratory cultures were analyzed anonymously to protect patient privacy.

TABLE 1 | Characteristics of Strains.

	Name	LVX[a] MIC (μg/ml)	LVX + EPI[b] MIC (μg/ml)	Amino Acid Change	Doubling Time, h (−/+ SEM)[c]
exoU	U-37	16	0.5	GyrA: T83I	2.0 (1.7–2.4)
	U-37PC*	16	0.5	GyrA: T83I ParC: S87L	1.7 (1.5–1.9)
	U-91	2	2	GyrA: T83I	3.9 (3.78–3.93)
	U-91PC*	16	2	GyrA: T83I ParC: S87L	3.9 (3.87–3.96)
	U-92	16	0.5	GyrA: T83I	2.5 (2.3–2.7)
	U-92PC*	16	0.5	GyrA: T83I ParC: S87L	2.8 (2.6–3.0)
exoS	S-139	4	0.5	GyrA: T83I	3.4 (3.3–3.6)
	S-139PC*	32	0.25	GyrA: T83I ParC: S87L	3.1 (2.9–3.3)
	S-247	16	1	GyrA: T83I	2.5 (2.4–2.6)
	S-247PC*	16	8	GyrA: T83I ParC: S87L	2.5 (2.4–2.7)
	S-215	16	1	GyrA: T83I	2.4 (2.2–2.7)
	S-215PC*	16	1	GyrA: T83I ParC: S87L	2.3 (2.2–2.5)

Mutants with Ser87Leu substitution in ParC denoted PC.* [a]*LVX, Levofloxacin;* [b]*EPI, efflux pump inhibitor;* [c]*Doubling time during exponential phase.*

Bacterial Strains and Culture Conditions

Strains of *P. aeruginosa* used in this study (**Table 1**) were selected from a collection of isolates obtained from the respiratory tract of hospitalized patients at Huntington Hospital, Pasadena, CA, USA from 2005 to 2009 and were stored in cryovials at −80°C in 30% glycerol. All strains had been previously characterized for: the presence of the *exoU* or *exoS* gene, resistance to levofloxacin, presence of mutations in the quinolone-resistance determining regions of *gyrA/B* and *parC/E*, and clonal relatedness by RAPD PCR, as described in Agnello and Wong-Beringer (2012). Strains were routinely grown as 5 ml cultures in Luria-Bertani (LB) broth in 12 ml polypropylene culture tubes at 37°C and shaking at 250 rpm for liquid culture, or grown on *Pseudomonas* Isolation Agar (PIA) plates at 37°C. Strains were routinely sub-cultured twice after inoculation from the frozen stock by 1:100 dilution (vol:vol) before use in any experiment.

Susceptibility Testing and Sequencing

Susceptibility testing to levofloxacin and rifampicin was performed by broth microdilution in twofold dilutions at concentrations ranging from 0.25 to 128 μg/ml according to guidelines recommended by CLSI (Clinical and Laboratory Standards Institute, 2007). In order to assess the involvement of the multidrug Mex efflux pumps to resistance, MIC of levofloxacin was also measured with the addition of an efflux pump inhibitor (EPI, MC-0228; Sigma) at 20 μg/ml (Kriengkauykiat et al., 2005).

For sequencing, genomic DNA was extracted and purified from isolates using the DNeasy Mini Kit (Qiagen). The quinolone-resistance determining regions of target genes *gyrA, gyrB, parC,* and *parE* were amplified by PCR using previously published primers and conditions and sequenced to identify mutations compared to wild-type strain PAO1 (**Table 2**) (Jalal and Wretlind, 1998; Lee et al., 2005; Winsor et al., 2011).

Creation of Target Site Mutants

Since most FQ-resistant isolates have *gyrA* mutations, three *exoU* and three *exoS* isolates with a pre-existing *gyrA* FQ-resistance mutation (Thr83→Ile substitution) were selected for mutagenesis. A site-directed point mutation in the *parC* gene leading to the amino acid substitution Ser87→Leu was introduced via electroporation of an oligonucleotide in order to create isogenic mutants, using the technique of oligonucleotide recombination as described previously (Swingle et al., 2010; Agnello and Wong-Beringer, 2014). Briefly, a single-stranded oligonucleotide 60 bases in length was designed using the *parC* gene sequence of reference strain PAO1 (Winsor et al., 2011) (**Table 2**). The oligo is identical to the PAO1 sequence from nucleotides 230 to 289, save for the point mutation TCG→TTG at location 31 of the oligo, corresponding to nucleotide 260 in the *parC* gene. This point mutation, which is the most common *parC* mutation observed among fluoroquinolone-resistant clinical strains, gives rise to the Ser87→Leu amino acid change in the ParC protein.

Strains were made electro-competent by several washes with 300 mM sucrose solution as described in Choi et al. (2006) and transformed by electroporation at 2.5 kV in a 0.2 cm cuvette using a Micropulser (Bio-Rad, Hercules, CA, USA). SOC medium (1 ml) was immediately added and the cells were outgrown overnight at 37°C on PIA plus levofloxacin at concentrations two and fourfold above the original minimum inhibitory concentration (MIC) of the isolates for selection. Single colonies were collected and the *parC* gene was sequenced as described above to confirm that recombination had occurred.

Growth Rate Measurements

Independent overnight cultures were diluted to OD_{600} ∼0.1 and grown at 37°C with shaking in 50 ml LB broth in a 100 ml flask. An aliquot of the culture (150 μl) was sampled every 30 min for 8 h and turbidity was measured at OD_{600} using a Tecan Sunrise microplate reader (Tecan Group Ltd., Switzerland). Results were reported as an average of at least three independent experiments in terms of doubling time per hour.

Insertion of Fluorescent Tag

Mini-Tn7 vectors developed and generously shared with us by Dr. Herbert Schweizer (Choi et al., 2005) were utilized to insert cassettes containing the genes encoding YFP or CFP as well as a cassette containing the *lux* operon into strains used for competition experiments and supercoiling experiments, respectively. The cassette inserts into the *P. aeruginosa* genome at a single location (*att*Tn7 site downstream of the *glmS* gene) and contains the gene for the fluorescent protein under the control of a constitutive promoter or the *lux* operon under the control of a supercoiling-sensitive promoter (Moir et al., 2007), as well as a gentamicin-resistance marker for selection. The delivery plasmid was co-electroporated with a helper plasmid (pTNS3) encoding the necessary transposase function for insertion. The electroporation protocol used for

TABLE 2 | Genetic elements used.

	Sequence	Reference
Primer name PCR		
glmS-up	CTGTGCGACTGCTGGAGCTGA	Choi and Schweizer, 2006
glmS-down	GCACATCGGCGACGTGCTCTC	Choi and Schweizer, 2006
Tn7-R	CACAGCATAACTGGACTGATTTC	Choi and Schweizer, 2006
Tn7-L	ATTAGCTTACGACGCTACACC	Choi and Schweizer, 2006
Oligo for recombination		
PC*	TGCTCGGCAAGTTCCACCCGCACGGCGACTTGGCCTGCTA CGAGGCCATGGTGCTGATGG	Agnello and Wong-Beringer, 2012
Plasmid	**Function**	**References**
pTNS3	Helper plasmid, encodes Tn-7 transposition pathway	Choi et al., 2005
pUC18T-mini-Tn7T-Gm-eyfp	Delivery plasmid for Gm^R marker and YFP	Choi and Schweizer, 2006
pUC18T-mini-Tn7T-Gm-ecfp	Delivery plasmid for Gm^R marker and CFP	Choi and Schweizer, 2006
TOP10/mini-Tn7-*PA0614* promoter-Gm-luxCDABE	Delivery plasmid for Gm^R marker and *lux* operon	Choi and Schweizer, 2006

transformation is described in detail in Choi and Schweizer (2006); briefly, strains were made electro-competent through a series of washes with 300 mM sucrose, subjected to electroporation as described above, and plated on LB plates containing 30 μg/ml gentamicin for selection. To confirm insertion had occurred, PCR was performed to amplify the insertion region using primers listed in **Table 2** and protocol as previously described (Choi and Schweizer, 2006) (data not shown).

In vitro Competition Assays

To investigate the effects of the *parC* mutation (PC*) on fitness, mutants were directly competed against isogenic parent strains in co-culture. Strains were tagged with either CFP or YFP for differentiation as described above. PC* mutant strains were initially tagged with CFP, and competed against the respective isogenic parent strain tagged with YFP. To confirm neutrality of the tags, experiments were also performed with YFP-mutants and CFP-parents.

Strains were grown overnight in LB, and 10^5 CFU of each strain was co-inoculated into 10 ml LB in a 50 ml flask, and grown with shaking at 37°C. Every 24 h, 10 μl of the culture was transferred to a new flask containing 10 ml fresh LB, and a sample of the culture was serially diluted then plated on PIA for CFU enumeration. Colonies of each color were counted using a fluorescent wide-field microscope (Zeiss Axio Zoom.V16). Experiment duration ranged from 4 to 7 days. The ratio of the number of CFUs of the mutant-to-parent strain on each day of the experiment was calculated and used to determine the relative fitness of the mutant. Experiments were repeated a minimum of five times. Assays were also performed with the addition of levofloxacin equal to 1/8 the MIC (2 or 0.5 μg/ml). At day 7 of each competition experiment, colonies were selected from plates, inoculated into LB, grown overnight, and stored at −80°C in 30% glycerol until ready for use in subsequent experiments. These were described as 'aged' strains. Aged strains were subjected to a secondary competition experiment vs. the original strains. Secondary competition with aged strains was performed as described above, and lasted for 4 days.

Supercoiling Assay

We adapted a reporter assay to estimate the ability to maintain supercoiling levels in mutants compared to parent strains by inserting a Tn7 cassette containing the *lux* operon under the control of a supercoiling-sensitive *P. aeruginosa* promoter from the gene *PA0614* (Moir et al., 2007). The cassette was inserted as described above. Luminescent strains were grown to mid-exponential phase, then diluted 1:4 in LB+ levofloxacin at 1/4, 1/2, and 1x the measured MIC, and grown in triplicate in a deep well 96-well plate for 7 h. Luminescence was measured using the Envision Multi-Label plate reader (Perkin-Elmer) as well as OD_{600} as described above. Relative Luminescence Units (RLU) was normalized to OD_{600} for all comparisons.

Mutation Frequency Assay

Mutation frequency was estimated during competition experiments using the spontaneous rifampicin resistance method (Oliver et al., 2000). At each 24-h interval, competition cultures were plated on PIA+ rifampicin at 5x the MIC (ranging from 40 to 500 μg/ml depending on the strain) in addition to plating on PIA. The mutation frequency reported is the number of colonies that grew on PIA + rifampicin after 48 h divided by the number of colonies on PIA alone. Colonies of each strain were differentiated and counted based on fluorescence as described above. For strain 139, 5x the MIC of rifampicin corresponds to 500 μg/ml, which turned the plates a dark shade of red and prevented the differentiation of the fluorescence of the colonies. Thus, for this strain, each rifampicin-containing plate was replica-plated using sterile velvets onto a fresh PIA plate and incubated for an additional 24 h before colonies were counted.

Statistical Analysis

GraphPad Prism v.6 (GraphPad.com, San Diego, CA, USA) was used to perform statistical analysis. ANOVA was used to compare continuous variables where appropriate, and student's

t-test was used to compare mean ratios to the null hypothesis of 1 (indicating no difference). A p value ≤ 0.05 denotes significance.

RESULTS

We previously created isogenic mutants from 6 clinical isolates of *P. aeruginosa* (three *exoU* and three *exoS*) by inserting a point mutation commonly observed in fluoroquinolone-resistant isolates into the *parC* gene (Agnello and Wong-Beringer, 2012). The goal of the current study was to investigate the biological effects of this target site mutation in a controlled genetic background. Mutants are denoted PC*. Interestingly, the addition of the *parC* mutation increased the levofloxacin MIC in only two of the six isolates. Characteristics of mutant and parent strains used in this study are summarized in **Table 1**.

Growth Rate in Monoculture

As an initial comparison of the relative fitness of mutant vs. parent strains, we tested the doubling time in independent cultures by measuring optical density every 30 min over an 8-h period; results are an average of at least three independent experiments (**Table 1**). Doubling time during exponential phase was calculated by dividing the time interval over the number of generations. Growth rates of all six mutants were comparable to parent strains.

parC Mutation Differentially Affects Competitive Fitness of *exoU* vs. *exoS* Strains

To investigate the fitness costs of the *parC* mutation, we performed head-to-head competition assays. Each PC* mutant was directly competed against its isogenic parent strain by growth in co-culture for 4 days. Because the strains competed are identical save for the point mutation, any fitness cost is attributable to the mutation. In each individual competition experiment, one strain is tagged with CFP (cyan fluorescent protein), and the other with YFP (yellow fluorescent protein). This allows for the direct enumeration of the number of colonies of each strain after serial dilution onto agar plates. Colonies were counted every 24 h, and the relative fitness of the mutant is determined from the ratio of mutant-to-parent CFU/ml. Each experiment was independently repeated at least five times. We confirmed the neutrality of the YFP and CFP tags by repeating each experiment with each strain carrying the opposite tag. No difference was found, and therefore results from all experiments were combined.

Figure 1 shows the average mutant-to-parent ratios over time for each strain. Each strain has a unique pattern of fitness costs associated with this mutation; however, in general, there appears to be a higher fitness cost for the *exoS* strains. The *exoS* strains S-139PC*, S-247PC*, and S-215PC* show a consistent fitness defect over all time points, with strain S-139PC* the most affected. On the other hand, strain U-92 has the smallest fitness effect, with the mutant/parent ratios ranging from 1.2 to 2.0 over 2–4 days of competition. Strain U-37 shows a similar pattern except the mutant starts with lower fitness (ratio 0.5 at day 2), but catches up to the parent strain by day 4 with a ratio of 0.99. Strain U-91 is unique and interestingly shows a striking increase in fitness due to the mutation; the mutant rapidly outcompetes the parent strain and completely takes over the culture by day 3.

exoU-PC* Mutants can Better Maintain Wild-Type Supercoiling Levels than *exoS*-PC* Mutants

We assessed supercoiling of parent vs. mutants in *exoU* strains U-92 and U-37 and *exoS* strains S-215 and S-139 by inserting a Tn7 genetic element in which a supercoiling sensitive promoter controls the *lux* operon (Moir et al., 2007). Due to challenges inserting the reporter, only two strains of each background were chosen as representative strains. Strains with the insertion were grown under levels of levofloxacin at and below the MIC in order to maximally induce expression of the reporter, and luminescence values were normalized to OD_{600}. We measured the level of luminescence in the parent strain as an indirect reporter of the baseline level of supercoiling; any difference in luminescence expression for the PC* strain reflects a change in supercoiling due to the *parC* mutation. Results show that the PC* mutants in general have decreased luminescence compared to parent strains; however, this decrease was significantly more pronounced in *exoS*-PC** strains (**Figure 2**), suggesting that *exoS* strains with the PC* mutation are somehow less able to maintain the supercoiling level of the parent strains, which may have negative effects on fitness. This is surprising given that the primary role of topoisomerase IV (ParC) is decatenation of daughter replicons and not regulation of supercoiling (Hooper, 2001); however, in this context it may suggest an overall difference in supercoiling regulation in *exoU* vs. *exoS* strains.

Analysis of Aged Strains

Because, we observed that the fitness of *exoU*-PC* mutants tended to increase after 24 h of competition, we investigated whether stable changes were occurring during competition that allowed the strains to compensate for fitness costs associated with the *parC* mutation. We collected colonies of parent and PC* strains from a primary competition experiment (parent vs. mutant) after 7 days and labeled these 'aged' strains. In addition, we investigated the effect of sub-inhibitory levels of fluoroquinolone exposure on competitive fitness and ability to compensate for fitness costs by collecting aged strains from primary competition experiments in which 1/8 MIC of levofloxacin was added to the media.

We were not able to collect aged colonies from the primary competition experiments of strain 91 due to the vast difference in fitness between the PC* mutant and parent strains; the PC* strain completely overtook the culture by day 3. Because this left us with only two *exoU* strains (U-92 and U-37), we chose two *exoS* strains in order to have an equal number in each group for comparison. We chose strains 215 and 139 because these represent both ends of the spectrum in terms of fitness of the PC* mutants *in vitro*; strain S-215PC* shows the least fitness cost of

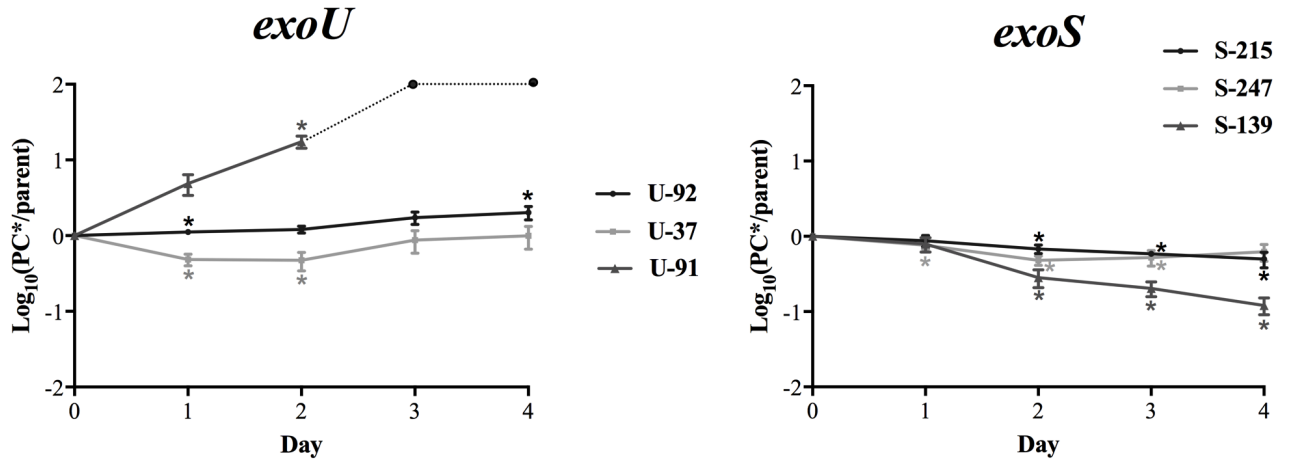

FIGURE 1 | *In vitro* fitness of *exoU* and *exoS*-PC* mutants. The average $\log_{10}$ CFU/ml ratio of PC* vs. parent strains per day of *in vitro* competition are shown. Ratios above zero indicate the mutant is more fit, while ratios below zero indicate a fitness cost. The ratio for *exoS* strains generally decreases over the course of the experiment, while the ratios for *exoU* strains either increases or remains stable. Results are an average of at least five independent experiments. Error bars = SEM. For strain U-91, the dotted line and circles indicate ratios after day 2 are beyond the limit of detection. *Indicates the PC*/parent CFU ratio is significantly different than 1 at $p < 0.05$.

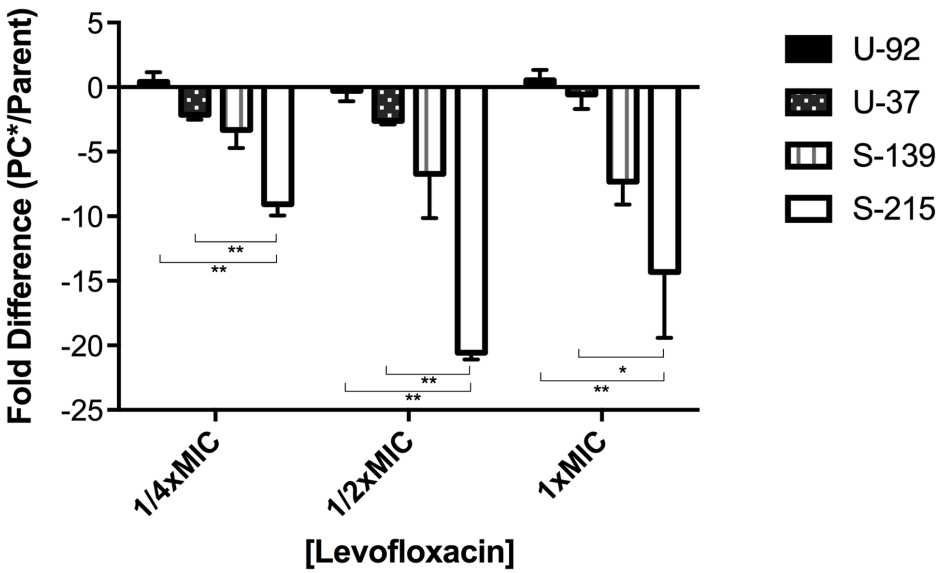

FIGURE 2 | Supercoiling changes in PC* mutants compared to parent strains. Each strain contains a chromosomal reporter construct in which the *lux* operon is under the control of a supercoiling-sensitive promoter. Strains were grown in concentrations of levofloxacin equal to 1/4, 1/2, and 1x the MIC. Luminescence was normalized to cell count using OD_{600}. Results are an average of three independent experiments, and error bars represent SEM. *$p < 0.05$; **$p < 0.01$.

all the *exoS* strains, while strain S-139PC* exhibits the greatest fitness cost.

Sequencing the FQ-target site genes of the aged strains revealed that no changes occurred in the genes associated with FQ-resistance except for *exoU* strain U-92, in which the parent acquired the Ser87Leu substitution in ParC during competition under levofloxacin exposure (+LVX) at 1/8 MIC. All PC* strains maintained the *parC* mutation. The MIC to levofloxacin did not change in any of the aged strains (data not shown).

Aged PC* Mutant *exoU* vs. *exoS* Strains Show Dramatic Difference in Fitness

To investigate whether the aged strains had acquired adaptations during competition that rendered them more fit, we compared the fitness of each aged strain to its un-aged counterpart. Aged strains were subjected to a secondary competition experiment in which they were competed against the original, un-aged strain that contained the opposite fluorescent tag, enabling the discrimination and enumeration of each strain during competition. Results reflect the aged-to-un-aged ratio at Day

2 (**Figure 3**). Because each aged strain was competed directly against an un-aged version of itself, we were able to directly observe if fitness had changed in the aged strains. All parent strains had a negligible change in fitness. Both *exoU*-PC* aged strains increased in fitness greater than fourfold, while the *exoS*-PC* aged strains showed a 50% decrease in fitness; S-139PC* was greater than 100 times less fit than before aging.

We also investigated whether growth under sub-inhibitory levels of levofloxacin affected fitness, and if mechanisms to compensate for fitness costs evolved under these conditions. The addition of levofloxacin did not affect the results of primary competition experiments (data not shown), but notable results were seen in the secondary competition experiments of the aged vs. un-aged strains, which also included 1/8 MIC of levofloxacin. Upon subsequent exposure to the drug, *exoU*-PC* strains were much more fit, outcompeting the un-aged strain rapidly. Specifically, the aged PC* mutants of *exoU* strains U-37 and U-92 outcompeted the un-aged strains significantly and rapidly, much more so than in the competition experiments without the drug. Interestingly, the measured MICs of the aged and un-aged strains remain unchanged. *exoS*-PC* strains showed conflicting results; S-215PC* had gained fitness, while S-139PC* had slightly decreased fitness (**Figure 3**).

Mutation Frequency

Compensation for potential fitness costs of a resistance-conferring mutation can evolve through the accumulation of beneficial mutations. Therefore, we investigated whether the rate of spontaneous mutation frequency could explain the increase in fitness during competition that was seen in *exoU*-PC* strains. Mutation frequency was estimated by calculating the frequency of spontaneous resistance to rifampicin (Rif^R) (Oliver et al., 2000). We estimated mutation frequency during the primary competition experiments for *exoU* strains U-92 and U-37, and *exoS* strains S-215 and S-139 by plating the mixed competition culture on agar containing 5x the MIC of rifampicin at each time point (Days 1–7), and counting the number of colonies based on fluorescence of each strain that were able to grow on the rifampicin-containing plates compared to the overall CFU counts. At Day 7, the *exoU*-PC* strains have both a higher mutation frequency than the parent strains as well as a higher mutation frequency compared to Day 1. *exoS* strain S-215PC* had a lower frequency at Day 7 compared to its parent strain as well as a lower frequency compared to Day 1. Surprisingly, *exoS* strain S-139PC*, which is significantly less fit than its parent strain, has a twofold higher mutation frequency than the parent strain at Day 7. However, its mutation frequency at Day 7 decreased almost threefold from Day 1 (**Figure 4**). Therefore, mutation frequencies seem to generally correlate with fitness results.

DISCUSSION

Pseudomonas aeruginosa is considered one of six bacteria that pose an immediate threat to public health, according to the Infectious Diseases Society of America, due to its increasing prevalence, broad arsenal of virulence factors, and emergence of resistance to all available antibiotics (Boucher et al., 2009). In its recent report entitled "Antibiotic Resistance Threats in the United States" the Centers for Disease Control named *P. aeruginosa* a serious threat that requires prompt action and continual monitoring to prevent a worsening of the problem of resistance (Centers for Disease Control [CDC], 2013). Notably, resistance rates of *P. aeruginosa* to the once-effective fluoroquinolone (FQ) antibiotics are now greater than 30% in the United States, with many of those strains also multi-drug resistant (Rosenthal et al., 2012).

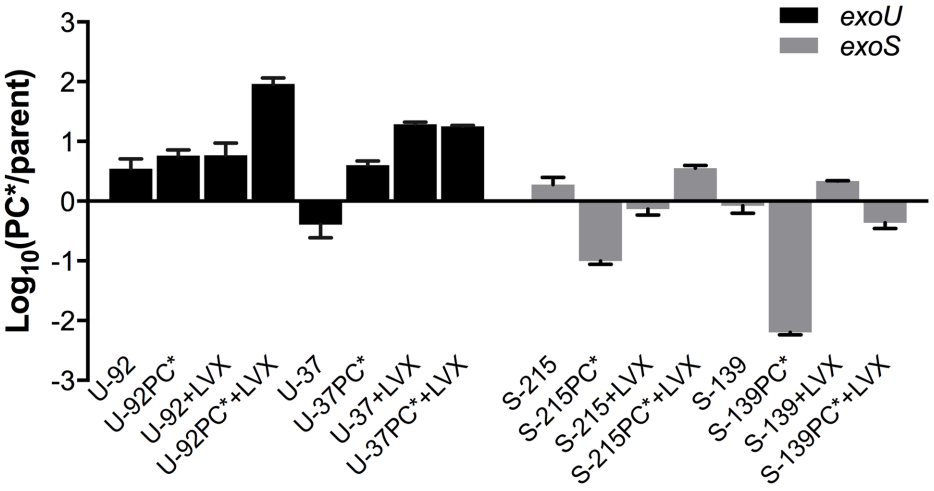

FIGURE 3 | Fitness of aged strains. Strains were collected at the end of a 7-day primary competition experiment (PC* vs. parent) with and without the addition of sub-inhibitory concentration of levofloxacin (+LVX) and subsequently re-competed in a secondary competition experiment (aged vs. un-aged) with or without levofloxacin at the same concentration. Results shown reflect the average fitness of aged strains after 48 h of competition. Experiments were performed in duplicate. Error bars = SEM.

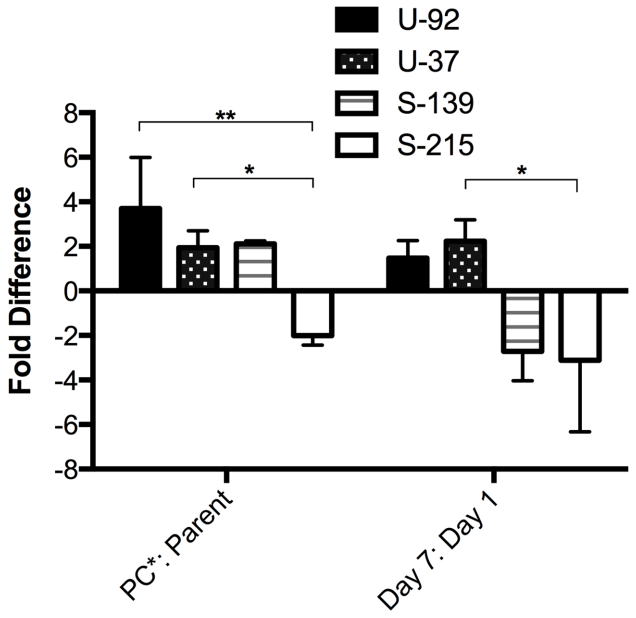

FIGURE 4 | Mutation frequency of PC* mutants vs. parent strains measured during competition experiments. The frequency of spontaneous rifampicin resistance was used to estimate mutation frequency at each time point during a 7-day competition experiment. Bars represent fold difference of the PC* vs. parent at Day 7 and the fold difference at Day 7 vs. Day 1 for each PC* strain. Error bars = SEM. *$p < 0.05$; **$p < 0.01$.

During acute disease, *P. aeruginosa* utilizes the toxins of the type III secretion system to circumvent the host immune system and establish infection. Of the four exotoxins, *exoU* is the most virulent, encoding a potent phospholipase that disrupts the plasma membrane and leads to rapid cell death (Sato et al., 2003). ExoU activity induces excessive inflammation and tissue damage in the host as well as increased bacterial dissemination that can lead to septic shock (Kurahashi et al., 1999) and higher rates of mortality compared to infections with ExoS-secreting strains (Shaver and Hauser, 2004; Zhang et al., 2014; Peña et al., 2015).

As the problem of antibiotic resistance continues to worsen, it is becoming apparent that the evolution of virulence factors and antibiotic resistance cannot be considered strictly independent processes (Beceiro et al., 2013; Hauser, 2014). Previously, we found that infection by FQ-resistant *P. aeruginosa* was an independent predictor for threefold higher mortality or prolonged illness by an additional 5 days compared to those infected with FQ-susceptible strains (Hsu et al., 2005). In a separate study, we reported that among patients from whom *P. aeruginosa* was isolated from the respiratory tract, strains with the combined traits of *exoU* and FQ-resistance were much more likely to cause pneumonia than *exoS,* FQ-resistant strains or either trait alone (Sullivan et al., 2014).

Analysis of 270 of our *P. aeruginosa* clinical isolates led to the observation that *exoU* strains are not only enriched in the FQ resistant sub-population, but are also more likely to have acquired two or more target site mutations than *exoS* strains, especially in the *parC* gene (Agnello and Wong-Beringer, 2012). In order to study the biological effects of a resistance-conferring mutation in a controlled, isogenic background, we adapted a technique for inserting point mutations into bacterial genomes for use in our clinical isolates (Swingle et al., 2010). Using the technique of oligonucleotide recombination, we created 6 FQ-resistant mutants (three *exoU* and three *exoS*) (Agnello and Wong-Beringer, 2014) from clinical strains by inserting a mutation into the *parC* gene.

Because the target site mutation, we inserted in *parC* rarely occurs in clinical isolates without a pre-existing mutation in *gyrA* (Jalal and Wretlind, 1998), we chose clinical strains that had a pre-existing *gyrA* mutation (Thr83Ile) in order to mimic FQ-resistant strains encountered in the clinical setting. These specific point mutations in *gyrA* and *parC* are commonly found in FQ-resistant clinical strains of *P. aeruginosa* (Nakano et al., 1997; Mouneimné et al., 1999; Higgins et al., 2003; Agnello and Wong-Beringer, 2012). The goal of the current study was to assess the biological effects on fitness of the *parC* mutation and determine if the magnitude of the effects differ in *exoU* vs. *exoS* strains.

The acquisition of resistance is generally thought to be accompanied by a fitness cost to the bacterium (Andersson and Levin, 1999); however, there are many reports of neutral fitness effects or enhanced fitness due to resistance-conferring mutations. Evidence from FQ-resistant *E. coli*, *S. pneumoniae, and N. gonorrhoeae* suggest that a secondary resistance mutation does not further decrease fitness and may restore the low fitness of primary mutants back to wild-type levels (Komp Lindgren et al., 2005; Rozen et al., 2007; Marcusson et al., 2009; Kunz et al., 2012; Machuca et al., 2014). Furthermore, many fitness effects seem to be strain dependent, as was shown in a study of ciprofloxacin-resistant *Campylobacter jejuni*, in which a *gyrA* mutation conferred a high cost to one strain but actually increased fitness for a different strain (Luo et al., 2005). Similar results were seen in *S. pneumoniae*, in which some FQ-resistant mutant strains had increased fitness while others had a significant cost (Balsalobre and de la Campa, 2008). This suggests that fitness effects are highly dependent on strain genetic background, which may explain the differences, we have seen between *exoU* and *exoS* strains.

Head-to-head competition assays are a standard method for investigating the relative fitness of a mutant strain compared to its isogenic parent strain, and it is possible to detect differences in fitness as small as 1% (Andersson and Levin, 1999; Corzett et al., 2013). Strains are mixed together in co-culture, and must compete for the limited available resources (Finkel, 2006; Kraigsley and Finkel, 2009). In most studies, strains are differentiated based on selective growth on antibiotic plates. However, since the *parC* mutation, we inserted did not increase the levofloxacin MIC in all strains, we had to develop a different approach. We took advantage of a Tn7-based system developed by Schweizer (Choi et al., 2005) in order to insert a cassette containing either YFP or CFP under the control of a strong promoter.

The growth of the PC* strains did not differ dramatically from those of the parents when grown in monoculture; however, differences were seen when strains had to compete for resources in the same culture flask during competition experiments.

Overall, the *parC* mutation imposed a moderate to considerable fitness cost on *exoS* strains, while *exoU* strains were able to better tolerate the mutation. Notably, fitness of *exoU*-PC* strains ranged from maintaining the wild-type level of fitness to outcompeting the parent strain by more than 10-fold, whereas *exoS*-PC* strains were consistently less fit than parent strains.

The FQ target site mutation investigated in the current study occurs in a topoisomerase enzyme and therefore may have an effect on the regulation of supercoiling. Common methods for investigating supercoiling levels rely on reporter plasmids that need to be selected for and maintained. Because, we are using clinical isolates, there is a high level of multi-drug resistance that precludes the use of standard selection antibiotics. To circumvent this, we inserted a Tn7 element (Moir et al., 2007) onto the chromosome in which a supercoiling sensitive promoter controls *lux* expression. *exoU*-PC* mutants were better able to maintain the supercoiling levels of the parent strains, while *exoS*-PC* mutants showed a more drastic change in supercoiling, as observed by decreased *lux* expression. Other studies have shown that changes in supercoiling are correlated with changes in fitness (Kugelberg et al., 2005; Marcusson et al., 2009), and therefore the regulation of supercoiling may reflect the level of overall fitness and may explain the fitness differences seen in our strains. The ability of *exoU*-PC* strains to better regulate supercoiling levels under the stress of FQ exposure reflects increased fitness of these strains overall. The supercoiling level of the cell affects global gene expression and replication efficiency, and changes in supercoiling may alter response to environmental stressors, or even modulate pathogenesis in the host (Redgrave et al., 2014).

In the primary competition experiments, *exoU*-PC* mutants tended to increase in fitness over the course of the 4-day experiment. This suggests that *exoU* strains may be acquiring beneficial adaptations that allow for the compensation of the fitness costs associated with the PC* mutation. Bacteria have the ability to rapidly evolve compensatory mechanisms to mitigate the fitness costs associated with antibiotic resistance, and compensatory mechanisms can reverse fitness costs without any loss of resistance (Andersson and Hughes, 2010). The *exoU*-PC* strains that had been 'aged' through a primary competition experiment for 7 days have increased fitness compared to the un-aged strains, while aged *exoS*-PC* strains have decreased fitness. The results suggest that the compensatory mechanisms in *exoU* strains most likely are acting as a repressor of the negative fitness effects of the *parC* mutation, as opposed to just conferring a general gain in fitness, based on the observation that the aged parent strains did not show as much of an increase in fitness as the PC* mutants. The observed ability of *exoU* strains to compensate for the costs of FQ-resistance has many clinically negative consequences. Compensation in clinical populations leads to the stabilization of resistant populations without the presence of drug (Andersson and Hughes, 2010). Increased fitness after acquisition of FQ-resistance adds to the already complicated problem of treating infections caused by highly virulent *exoU* strains.

The inability of *exoS*-PC* strains to regulate supercoiling suggests that the compensatory mechanisms in *exoU* strains are acting to maintain wild-type supercoiling levels in the PC* mutants, as has been reported in other studies of compensation of the costs of FQ-resistance (Kugelberg et al., 2005; Marcusson et al., 2009). Although, we did not identify the exact mechanisms responsible for the compensatory effects, we have shown that they are stable since strains were frozen and grown before testing for compensation in secondary competition experiments, and the results were reproducible in repeated experiments. Sequencing of the quinolone-resistance determining regions of *gyrA/B* and *parC/E* revealed no additional mutations had occurred during competition; however, beneficial mutations may have occurred elsewhere. For both *exoU* strains tested, the mutation frequencies correlated with the increase in fitness observed during competition experiments, suggesting that higher mutation frequency may facilitate the acquisition of beneficial mutations that mitigate the fitness costs of the *parC* mutation.

A dramatic increase in fitness occurred in the aged *exoU*-PC* strains taken from a primary competition experiment in which sub-inhibitory levels (1/8 the MIC) of levofloxacin were added to culture. Although this low level of drug did not affect the growth of strains in the primary competition experiment, when the aged *exoU*-PC* strains were re-competed under the same concentration of levofloxacin, they were 20–100 times more fit than before aging. In contrast, *exoS*-PC* strains were dramatically less fit under these circumstances. However, none of the aged PC* strains had an increase in MIC, nor any additional FQ-resistance mutations in target site genes. These results suggest that although the level of levofloxacin was much below the MIC, highly fit strains were selected for rapidly, more so than in conditions without drug. Also, the highly fit strains were not more resistant, suggesting that perhaps the presence of levofloxacin accelerated the process of compensation for the already present resistance mutations. As sub-inhibitory concentrations of antibiotics are routinely present during treatment due to insufficient dosing or inadequate penetration to certain areas of the body (Baquero and Negri, 1997; Andersson and Hughes, 2014), the implications of our results are alarming and suggest that low levels of antibiotic can rapidly select for highly fit strains, preferentially the highly virulent *exoU*-containing strains. Furthermore, when these highly fit, highly virulent strains are re-introduced to the antibiotic, they will rapidly outcompete all other strains. The sub-inhibitory concentration was also able to select for a *parC* mutation in an *exoU* parent strain, emphasizing the known phenomenon that selection for resistance mutations can occur at sub-inhibitory levels of antibiotic (Baquero et al., 1998; Andersson and Levin, 1999; Gullberg et al., 2011; Andersson and Hughes, 2014).

Overall, these results suggest a potentially lower fitness burden associated with FQ-resistance for *exoU* strains than for *exoS* strains, which in part provides an initial biological explanation for *exoU* strains' greater tendency to acquire FQ-resistance in the clinical setting. Although, we have yet to identify the specific genetic components underlying the fitness differences, we suspect that genes unique to strains with the *exoU* genetic background may contribute to the fitness benefits seen in this study. The *P. aeruginosa* genome consists of a highly conserved core genome, but variability is introduced in the form of genomic islands, which make up the accessory genome. Genes within the islands usually encode accessory activities such as specific

pathogenicity or symbiosis factors (Harrison et al., 2010). The *exoU* gene, along with its chaperone *spcU*, is located on a pathogenicity island, a specialized genomic island. *exoU* has been identified as part of a few different pathogenicity islands. The most highly studied is from the reference strain PA14, in which the *exoU* gene is on an island named PAPI-2 (Kulasekara et al., 2006). Studies with PA14 have shown that virulence is dependent on the presence of the entire island and not just the *exoU* gene alone; therefore, other as yet unknown genes contained on the pathogenicity islands contribute combinatorially to the increased virulence of *exoU* strains (Harrison et al., 2010). Furthermore, pathogenicity in strain PA14 requires the coordinated action of multiple virulence factors, associated with both the core and accessory genomes (Lee et al., 2006). Therefore, it is possible that other genes in the accessory genome in combination with *exoU* may provide fitness benefits to *exoU* strains that allow for increased ability to adapt to the fitness costs of FQ-resistance. Genomic islands have been shown to confer fitness benefits, and the accessory genome of *P. aeruginosa* is an important driver of the ability of strains to persist in a particular environment (Hacker and Carniel, 2001; Ozer et al., 2014).

This study has several limitations. We acknowledge that a limited number of clinical strains and their isogenic mutants were evaluated in the study. Despite having access to a large collection of clinical strains, the selection of strains to create isogenic target site mutants to study the effect of FQ-resistance on fitness proved challenging, as most of the isolates in our collection were found to already contain the resistance-conferring mutation that, we are investigating, due to the high prevalence of FQ resistance in clinical isolates of *P. aeruginosa*. Therefore, we were not able to use those to create isogenic pairs. Furthermore, genetic manipulation of these clinical isolates proved to be difficult, as these strains were not universally amenable to the creation of isogenic mutants via oligonucleotide recombination. Nonetheless, it is worth nothing that the strains evaluated in this study were carefully selected to represent a broad range of characteristics such as varying degrees of FQ resistance, virulence, and clinical outcomes. While the limited number of strains evaluated in this study may not necessarily represent the genetic variability present among all *exoU* and *exoS* clinical strains, strain-specific differences in fitness effects were observed even among strains with the same *exoU* or *exoS* genetic background, though the trend observed appears to follow similar pattern for strains with the same *exoU* or *exoS* genetic background.

Understanding the fitness costs of antibiotic resistance and possibilities of compensation for these costs is essential for rationally combating the problem of antibiotic resistance. Importantly, this study outlines a useful model with the creation of isogenic clinical strains for investigating the biological costs of resistance in a medically important pathogen and can be applied to other drug-organism pairs.

AUTHOR CONTRIBUTIONS

MA, AW-B, and SF designed the experiments. MA and AW-B wrote the manuscript. SF edited and provided critical review of the manuscript.

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Virulence and Genomic Feature of Multidrug Resistant *Campylobacter jejuni* Isolated from Broiler Chicken

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The aim of this study was to reveal the molecular mechanism involved in multidrug resistance and virulence of *Campylobacter jejuni* isolated from broiler chickens. The virulence of six multidrug resistant *C. jejuni* was determined by *in vitro* and *in vivo* methods. The *de novo* whole genome sequencing technology and molecular biology methods were used to analyze the genomic features associated with the multidrug resistance and virulence of a selected isolate (*C. jejuni* 1655). The comparative genomic analyses revealed a large number of single nucleotide polymorphisms, deletions, rearrangements, and inversions in *C. jejuni* 1655 compared to reference *C. jejuni* genomes. The co-emergence of Thr-86-Ile mutation in *gyrA* gene, A2075G mutation in 23S rRNA gene, *tetO*, *aphA* and *aadE* genes and pTet plasmid in *C. jejuni* 1655 contributed its multidrug resistance to fluoroquinolones, macrolides, tetracycline, and aminoglycosides. The combination of multiple virulence genes may work together to confer the relative higher virulence in *C. jejuni* 1655. The co-existence of mobile gene elements (e.g., pTet) and CRISPR-Cas system in *C. jejuni* 1655 may play an important role in the gene transfer and immune defense. The present study provides basic information of phenotypic and genomic features of *C. jejuni* 1655, a strain recently isolated from a chicken displaying multidrug resistance and relatively high level of virulence.

Keywords: *Campylobacter jejuni*, broiler chicken, multidrug resistance, virulence, *de novo* genome sequencing

INTRODUCTION

Campylobacter jejuni is one of the most important foodborne pathogens worldwide (Abril et al., 2010). The macrolides (mainly erythromycin and azithromycin) and fluoroquinolones are the empirical drugs of choice for treatment of campylobacteriosis (Allos, 2001). Other antimicrobial agents, including gentamicin, meropenem, and clindamycin are alternative therapies (Iovine et al., 2008).

The National Antimicrobial Resistance Monitoring System (NARMS) in the USA and Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) data showed that multidrug resistance in *C. jejuni* has been very rare (0.3-0.7%) from retail chicken meat (DANMAP, 2014; NARMS, 2015). Multidrug resistant *C. jejuni* have been isolated from chicken

farms in China recently (Chen et al., 2010; Hao et al., 2015; Wang et al., 2016). The multidrug resistant *C. jejuni* in chicken may greatly threaten food safety and human public health, therefore it is important to investigate the virulence potential and mechanism involved in multidrug resistance and virulence of *C. jejuni*.

Although genomes of many human-source *C. jejuni* (e.g., NCTC11168, 81–176, 260.94, HB93-13, CF93-6, and 269.97) have been sequenced, only three genomes of *C. jejuni* (305, RM1221, and 81-176-DRH212) from poultry have been obtained (Parkhill et al., 2000; Fouts et al., 2005; Hofreuter et al., 2006). *C. jejuni* RM1221 (ATCC BAA-1032) is a chicken isolate with unique lipoolgosaccharide and ability to colonize chicken skin (Fouts et al., 2005). *C. jejuni* 305 is a turkey isolate with stress tolerance (Takamiya et al., 2011). *C. jejuni* 81–176-DRH212 is a *C. jejuni* 81–176 variant with enhanced fitness in the chicken gastrointestinal tract (Johnson et al., 2014). The genome sequences of broiler *C. jejuni* isolates with multidrug resistance and high virulence have not been published previously. In order to investigate the mechanisms involved in the multidrug resistance and increased virulence in *C. jejuni* isolated from broiler chickens in China, the genomic profile of a *C. jejuni* isolate with relative higher virulence and multidrug resistance was determined by the *de novo* sequencing technology in the present study.

MATERIALS AND METHODS

Isolation and Identification of Chicken *C. jejuni*

Six strains (1442, 1447, 1614, 1622, 1655, and 1685) were isolated from caecum of healthy broiler chicken in the chicken farms located in the center of China in 2013. These farms have a long history of usage of different antimicrobial drug for prevention and treatment chicken disease. These strains were confirmed as *C. jejuni* by classic biochemical test and PCR amplification of the 16S rRNA, *mapA* and *vs1* genes (Stucki et al., 1995; Hao et al., 2013, 2015). The *C. jejuni* isolates were grown on Mueller-Hinton (MH) agar supplemented with 5% sheep blood at 42°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) for 24–48 h.

Antimicrobial Susceptibility Test

The minimum inhibitory concentrations (MICs) of erythromycin (ERY), tylosin (TYL), ciprofloxacin (CIP), enrofloxacin (ENR), doxycycline (DOX), tetracycline (TET), amikacin (AMK), and gentamicin (GEN) were determined using the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) M31-A3 guidelines (CLSI., 2008). The *C. jejuni* ATCC33560 was used as quality control for the MIC determination.

Preliminary Pathogenicity Test

Newly hatched broiler chickens were purchased from Zhengda Limited Company (Wuhan, China). Prior to the experiment, all the chickens tested negative for *C. jejuni* strains by *C. jejuni* isolation and identification methods. The chickens were randomly divided into 8 groups with 7 chickens in each group. The chickens in groups 1–7 were administrated once with 5×10^5 CFU of each *C. jejuni* strain (1442, 1447, 1614, 1622, 1655, 1685, and RM1221) by oral injection, respectively. The eight group served as the negative control with no bacterial infection. The clinical symptoms and mortality of the chickens in each group was observed daily. The colonization rate of each *C. jejuni* strain was determined using selective medium containing 32 αg/mL erythromycin.

Cytotoxin Assay

The murine macrophage RAW264.7 cell line was used to evaluate the cytotoxic effects of the six *C. jejuni* isolates. The tissue culture cytotoxin assay was carried out as previously described (Guerrant et al., 1987) with some modification. Briefly, *C. jejuni* was treated by 2000 μg/ml polymyxin B for 1 h at 37°C (Ashkenazi and Cleary, 1990) and centrifuged at 8000 rpm for 20 min. The supernatant were filtered through 0.22 μm filter. Purified shiga toxin (Chinese Center for Disease Control and Prevention) and 0.3% Triton X-100-PBS were used as positive controls, and polymyxin B-treated broth and polymyxin B-PBS (phosphate-buffered saline) were used as negative controls. Suspensions of murine macrophage RAW264.7 cells (100 μl; 5×10^5 cells) were placed in 96-well flat-bottom microtiter plates and allowed to adhere for 1 to 3 h to form the tissue culture monolayers. Serial twofold dilutions of the prepared *C. jejuni* filtrates were added to the tissue culture monolayers in 100 μl volumes. The monolayers were incubated with the filtrates at 37°C in 5% CO_2 for 24 h. The monolayers were then examined by phase-contrast microscopy for the percentage of cells rounded. Cell death was determined by trypan blue dye uptake (A540 value) and correlated with cell morphology after Giemsa staining. The index of cytotoxin effect was calculated by the formula.

$$100 \times \left[1 - \frac{A540(test) - A540(positive)}{A540(negative) - A540(positive)}\right].$$

Biofilm Assays

To measure the biofilm formation of the six *C. jejuni* strains, crystal violet staining was used as described previously for *C. jejuni* and other bacteria (Asakura et al., 2007; Fields and Thompson, 2008; McLennan et al., 2008; Reuter et al., 2010) with some modification. Briefly, the 200 αl of *C. jejuni* fresh culture (OD_{600} = 0.05) was added to 96-well flat-bottom microtiter plates. Plates were incubated without shaking at 42°C under microaerobic conditions for 24, 48, and 72 h. For crystal violet staining, each well was washed with PBS three times to remove the planktonic cells. The 200 αl of methanol were added and incubated for 15 min and then dried at room temperature. Then 200 αl of 0.1% Hucker crystal violet solution were added, and the plates were incubated at room temperature for 5 min. Unbound crystal violet was washed off with PBS, and the plates were dried at 60°C. Bound crystal violet was dissolved in 30% glacial acetic acid for 10 min. The absorbance was determined using a plate reader at 570 nm. The wells with sterile medium were used as blank control. The blank corrected absorbance values of *C. jejuni* strains were used for reporting biofilm production. Assays were repeated at least three times with three technical replicates.

LD_{50} Determination

Based on the results from the preliminary pathogenicity experiments with chickens, cytotoxin testing and biofilm assays, three *C. jejuni* isolates (1442, 1622, and 1655) were selected to further determine their median lethal dose (LD_{50}) using 2 days old chicken orally or intraperitoneally infected with a range of concentration (10^5–10^7 CFU) of each *C. jejuni* isolate. All the chickens tested negative for *C. jejuni* prior to inoculation using the *C. jejuni* isolation and identification methods described above. The chickens were randomly divided into six groups with 35 chickens in each group. The chickens in each group were orally or intraperitoneally administrated with 0, 10^5, 10^6, or 10^7 CFU of the *C. jejuni* isolates, respectively. Seven chickens were inoculated with each of the dilution of the test strains.

Adhesion and Invasion Assay

The adhesion and invasion of these three *C. jejuni* strains (1442, 1622, and 1655) was determined as previously described (Almofti et al., 2011a). Briefly, 5.0×10^7 CFU/mL of the *C. jejuni* strain was used to inoculated the monolayers of macrophage RAW264.7 cells at multiplicity of infection (MOI) of 100. The infected monolayers were incubated for 3 h to allow the occurrence of adhesion and invasion. For determination of the total number of adherent and internalized bacteria, the monolayers were washed three times with Dulbecco's Modified Eagle's Medium (DMEM) without antibiotic to remove the extracellular unbound bacteria. The monolayers were then lysed to release the intracellular bacteria. For determination of the invading bacteria, the monolayers were washed twice with aspirated DMEM medium and 100 mg/ml gentamicin was added for 1 h to kill the extracellular and bound bacteria. The monolayers were then washed three times and lysed to release intracellular bacteria. The number of adherent bacteria was obtained by subtraction of the internalized bacteria number from total number of adherent and internalized bacteria.

Intracellular Survivability Assay

To determine the intracellular survivability of *C. jejuni* within macrophage RAW264.7, the invasion period of each *C. jejuni* strain (1442, 1622, and 1655) was extended to 3, 6, 10, 16, 24, 48, and 72 h post-infection. Cells were washed and lysed as previously described in the adhesion and invasion assay. The surviving intracellular bacteria were enumerated by plating serial dilutions on blood agar and counting the resultant colonies.

Motility Test

To determine the migration of the three selected strains (1442, 1622, and 1655), a fresh culture of *C. jejuni* strain was inoculated into MH broth and cultured to their logarithmic growth phase. Then 3 αl of bacteria with consistent concentration were stabbed into 0.4 % MH agar. Plates were incubated at 42°C under microaerophilic conditions for 24 h and the motility was scored by measuring the diameter of the growth in each plate. The experiment was performed in triplicate.

De Novo Whole-Genome Sequencing Of *C. jejuni* 1655

Total genomic DNA of *C. jejuni* 1655 was extracted using the TIANamp Bacteria DNA kit according to the manufacturer's protocol. The genomic DNA was sent to Shanghai Biotechnology Corporation for the *de novo* whole genome sequencing performed in a HiSeq 2500 platform using a multiplexed, 2 × 100 nucleotide paired end approach (Illumina). The sequence analysis and assembly were carried out using CLC Genomics Workbench (ver. 6.0., Qiagen). Prediction and identification of the main components of the genome, including open reading frames (ORFs), tRNA, rRNA, ncRNA, CRISPR-Cas system, and repeat elements, were conducted using software of Glimmer 3.02 and some other online tools.

The nucleotides and predicted proteins of 1655 were compared with previously sequenced *C. jejuni* genomes in GenBank using updated nt and nr databases with BLAST software. The genomes used as reference sequences for the comparative genomic analysis were *C. jejuni* 81–176, *C. jejuni* NCTC11168, *C. jejuni* RM1221. The comparative genomic analysis was performed using BLASTN, BLASTX, Mummer and Quast. The sequence data from *C. jejuni* 1655 was compared to the results of antimicrobial resistance and virulence to identify genetic factors that correspond to the observed mechanisms.

Nucleotide Sequence Accession Numbers

The whole genome sequence of *C. jejuni* 1655 has been deposited at DDBJ/ENA/GenBank under the accession MDDM00000000.

Ethics Statement

All the experimental procedure in this study was performed according to the guidelines of the committee on the use and care of the laboratory animals in Hubei province China. The study was approved by Animal Ethics Committee of Huazhong Agricultural University (hzauch 2014-002) and the Animal Care Center, Hubei Science and Technology Agency in China (SYXK 2013–0044). These experiments were in line with national regulations about animal welfare ethics. All the animals were monitored throughout the study for any adverse effect signs. All efforts were made to minimize suffering of animals.

RESULTS

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) of different antimicrobial agents against the 6 *C. jejuni* isolates (1442, 1447, 1614, 1622, 1655, and 1685) were presented in **Table 1**. Each isolate exhibited resistance to all the tested drugs, while three (1622, 1655, and 1685) showed higher-level MICs to erythromycin and gentamicin than the others.

Pathogenicity of *C. jejuni* Isolates on Chicken

After oral infection with 10^5 CFU of the *C. jejuni* isolates, no clinical change was observed in two groups infected with 1442 or 1447, while the other four isolates (1614, 1622, 1655, and 1685) and RM1221 caused different degrees of diarrhea and bloody

TABLE 1 | MICs of different antimicrobial agents against six *C. jejuni* isolates (μg/mL).

Strains	MICs of different antimicrobial agents							
	ERY	TYL	ENR	CIP	TET	DOX	GEN	AMK
1442	128	>256	64	128	128	32	64	16
1447	64	>256	32	8	128	32	1	8
1614	256	>256	0.25	16	64	64	>256	4
1622	512	>256	256	128	>256	32	>256	64
1655	512	>256	32	64	256	32	>256	8
1685	512	>256	16	32	64	32	>256	16
RM1221	0.5	2.0	0.5	0.125	0.5	0.25	0.5	0.5

ERY, Erythromycin; TYL, Tylosin; ENR, Enrofloxacin; CIP, Ciprofloxacin; TET, tetracycline; DOX, Doxycycline; GEN, Gentamicin; AMK, Amikacin.

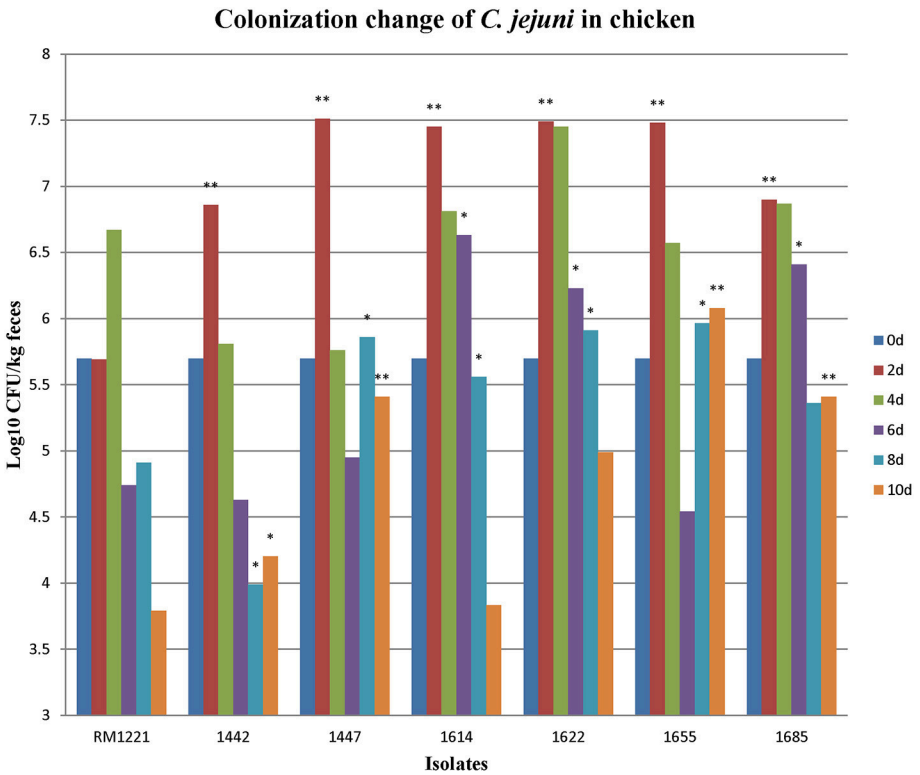

FIGURE 1 | Colonization change of *C. jejuni* isolates in chicken after inoculation. The Y-axis is the number (Log_{10} CFU/kg fece) of each strain colonizing the birds at different days. The asterisk (*) and (**) represent statistical significant difference with $P \leq 0.05$ and $P \leq 0.01$ comparing with *C. jejuni* RM1221, respectively.

stools, respectively. The clinical signs of three strains (1614, 1685, and RM 1221) were recovered at day 4, while serious diarrhea caused by 1622 and 1655 was sustained for over 8 and 10 days, respectively (Table S1). Therefore, 1655 exhibited relative higher pathogenicity on chickens than RM1221 and other five *C. jejuni* isolates. Within 15 days, the mortality of all the strains was 0% following oral inoculation of 2 days-old chicken.

Colonization of *C. jejuni* Isolates in Chicken

After oral administration with 5×10^5 CFU of the *C. jejuni* isolates to 2-days old chickens, all the strains could colonize chickens' intestinal tract. Fecal samples were obtained at different time points (2, 4, 6, 8, and 10 days). RM1221 colonized at concentration of 10^5–10^7 CFU/kg feces at day 2 and day 4, but the concentration was reduced to less than 10^4 CFU/feces at day 10 (**Figure 1**). The concentrations of five isolates (1442, 1447, 1622, 1655, and 1685) were higher than 10^4 CFU/kg feces within the ten days (**Figure 1**). Comparing with the colonization of RM1221, three isolates (1622, 1655, and 1685) exhibited significantly stronger colonization (**Figure 1**).

Cytotoxin of the Six *C. jejuni* Isolates in Macrophage Cells

Figure 2 showed the killing effect (index) of cytotoxin released by six strains (1442, 1447, 1614, 1622, 1655, and 1685) on murine

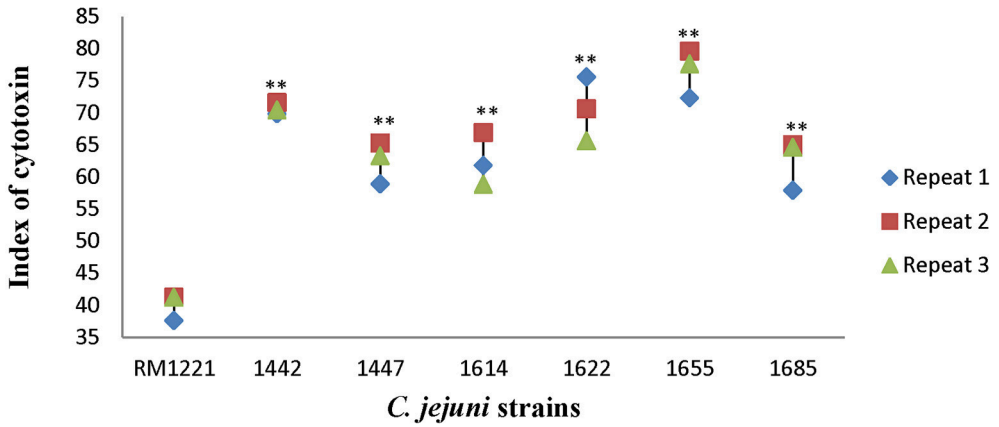

FIGURE 2 | Cytotoxic production of *C. jejuni* isolates in murine macrophage RAW264.7 cell. The Y-axis is the index of cytotoxic production of each strain. The results were obtained from three independent repeats. The asterisk (*) and (**) represent statistical significant difference with $P \leq 0.05$ and $P \leq 0.01$ comparing with *C. jejuni* RM1221, respectively.

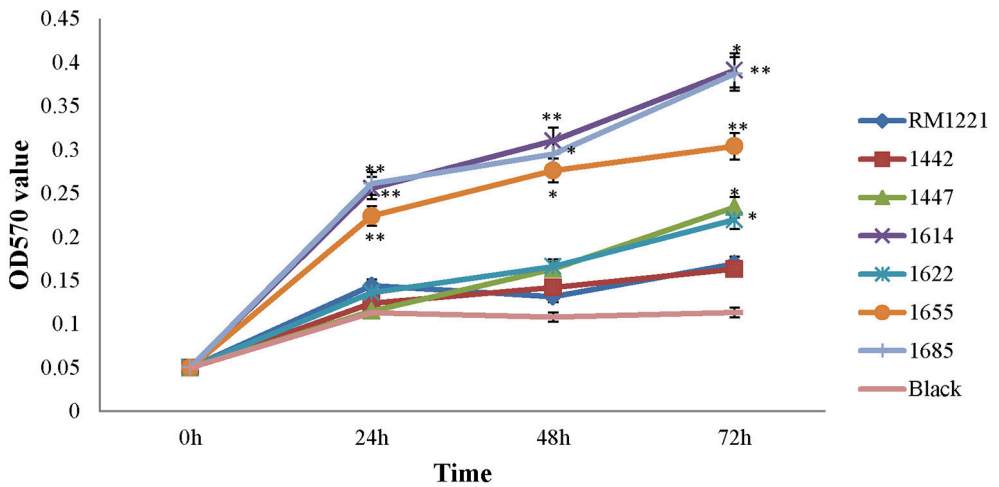

FIGURE 3 | Biofilm formation of *C. jejuni* isolates at different time points under ordinary conditions. The Y-axis is the OD_{570} value of crystal violet in biofilm. The results were obtained from three independent experiments. The asterisk (*) and (**) represent statistical significant difference with $P \leq 0.05$ and $P \leq 0.01$ comparing with *C. jejuni* RM1221, respectively.

macrophage RAW264.7. The killing effect of cytotoxin from all the strains was lower than 80%. However, compared to RM 1221, significant differences of the index of cytotoxin were observed in these *C. jejuni* strains. Among them, 1442, 1622, and 1655 seemed to release more cytotoxin and therefore exhibited higher killing effect on murine macrophage RAW264.7.

Biofilm Formation of the Six *C. jejuni* Isolates

Different level of biofilm formation was observed in each *C. jejuni* strain (**Figure 3**). It seemed that the level of biofilm formation had positive correlation with the incubation time. The highest level of biofilm formation was occurred after 72 h incubation (**Figure 3**). Compared to RM1221, a significant difference of biofilm formation was observed in three isolates (1614, 1655, and 1685) after each incubation time, indicating that these three strains may have stronger ability to form biofilm (**Figure 3**).

LD_{50} of the Three Selected *C. jejuni* Isolates on Chicken

From the preliminary pathogenicity testing on chicken, cytotoxin testing and biofilm assays, three isolates (1442, 1622, and 1655) were selected for LD_{50}determination. As the result shown in **Table 2**, different fatality rates were observed in different groups.

After oral infection, the highest concentration (3.70×10^7 CFU) of 1442 and 1622 could cause mortality rate of 0% (0/7) and 28.6% (2/7) respectively, while 3.7×10^7 CFU of 1655 cause fatality rate of 55.6% (4/7). For intraperitoneal infection, 3.7×10^6CFU of 1442 and 1622 only leaded to one chicken death, while the lowest concentration (3.7×10^5 CFU) of 1655 leaded to 100% death of chickens. The 1655 exhibited highest pathogenicity on chicken with the calculated LD_{50} of 8.45×10^7 CFU for oral infection and LD_{50} of less than 3.7×10^5 CFU for Intraperitoneal infection (**Table 2**).

TABLE 2 | LD_{50} of the three multidrug resistant strains on 2 days old chickens.

Infection route	Strains	Infection dose of (CFU)	Number of chickens	Number of Dead chicken	LD_{50}
Oral infection	1442	3.70×10^7	7	0	NA
		3.70×10^6	7	0	
		3.70×10^5	7	0	
		0	7	0	
	1622	3.70×10^7	7	2	NA
		3.70×10^6	7	0	
		3.70×10^5	7	0	
		0	7	0	
	1655	3.70×10^7	7	4	8.45×10^7
		3.70×10^6	7	1	
		3.70×10^5	7	1	
		0	7	0	
Intraperitoneal infection	1442	3.70×10^7	7	2	NA
		3.70×10^6	7	1	
		3.70×10^5	7	0	
		0	7	0	
	1622	3.70×10^7	7	1	NA
		3.70×10^6	7	1	
		3.70×10^5	7	0	
		0	7	0	
	1655	3.70×10^7	7	7	$<3.70 \times 10^5$
		3.70×10^6	7	7	
		3.70×10^5	7	7	
		0	7	0	

Adhesion and Invasion of *C. jejuni* on Macrophage RAW264.7

The three isolates (1442, 1622, and 1655) exhibited remarkable higher adhesion and invasion to macrophage cell RAW264.7 than RM1221 (**Figure 4**). The significant change of adhesion to macrophage cells ($P < 0.01$) was observed in 1622 and 1655. However, there was no significant difference in the adhesion and invasion between three selected isolates (1442, 1622, and 1655).

Intracellular Survivability of *C. jejuni* in Macrophage RAW264.7

The intracellular survivability of the three strains (1442, 1622, and 1655) was shown in **Figure 5**. Comparing with RM1221, three selected strains showed remarkable advantage in intracellular survivability in murine macrophage RAW 264.7. RM1221 had short survival time (24 h), while three strains were able to survive for more than 48 h, although a considerable decrease in the number of all internalized was observed. Among them, 1622 and 1655 exhibited highest survivability at 72 h. At each time point, the post-infection number of surviving 1655 was higher than 1622, suggesting that 1655 strain had strongest survivability.

Motility of the Three Selected *C. jejuni*

As shown in **Figure 6**, there was apparent difference between diameter growth rings of the three strains (1442, 1622, and 1655) on 0.4% MH agar plate. RM1221 and 1622 were not motile, while 1655 and 1442 had a strong motility.

Whole-Genome Features of *C. jejuni* 1655

Based on the above *in vitro* and *in vivo* studies, 1655 was selected for subsequent whole genome sequencing to analyze the potential genetic mechanisms involved in the multidrug resistance and high virulence.

The basic genome information of 1655 and other three reference strains (RM1221, 81–176, and NCTC11168) are summarized into **Table 3**. The 1655 genome was comprised of a chromosome and a tetracycline resistance plasmid pTet. The pTet plasmid of 1655 had 98% homology with pTet plasmid in 81176. The genome of 1655 was 1720, 061 bp long and contained 1733 predicted coding regions. The genomes of two chicken original strains (1655 and RM1221) were significantly larger than that of two human isolates (*C.* 81–176 and NCTC11168). The GC content of 1655 was 31.36%, consistent with the reference genome sequences.

The genome sequence of 1655 was composed by 35 large contigs (**Figure 7**). The cluster of orthologous groups (COG) assignment for predicted gene products were shown in the third circle from the outside in **Figure 7**. A total of 1733 putative gene products were assigned to COG identifications classified into 19 COG categories. Three copies of rRNA gene cluster (5s rRNA, 16s rRNA, and 23s rRNA) and 40 tRNA genes were identified on the *C. jejuni* 1655 chromosome.

The global alignment of four genomes (RM1221, 81–176, NCTC11168, and 1655) is shown in **Figure 8**. High similarity was observed in the genome of three reference strains (RM1221, 81–176, and NCTC11168). However, disordered distribution, deletions and rearrangements were observed in the genome of 1655 when compared with genome of three reference strains.

Resistance Determinants in *C. jejuni* 1655

The genome sequencing data showed that 1655 contained T86I mutation in the *gyrA* gene. Some mutations (e.g., A2075G mutation, C2113T, position 1732–1791 mutations) were found in the 23S rRNA of *C. jejuni* 1655. The ribosomal L4 contained V196A mutation and ribosomal L22 had A74G mutation. The tetracycline resistant gene *tetO* was located in pTet plasmid. The *C. jejuni* 1655 also contained *aphA* and *aadE* exogenous genes

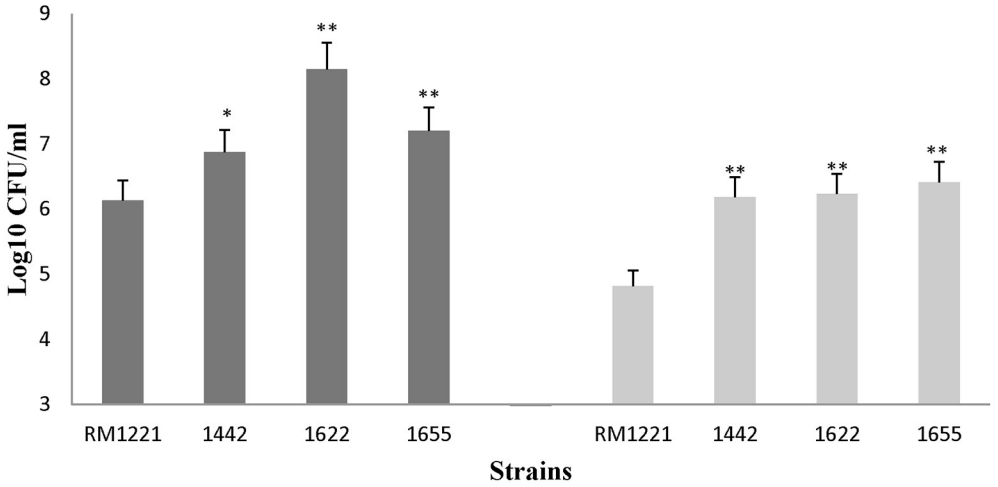

FIGURE 4 | Adhesion (left) and invasion (right) of *C. jejuni* strains in macrophage RAW264.7 cell. The Y-axis is the mean of log10 CFU/mL of each strain in the cells. The results were obtained from three independent experiments. The asterisk (*) and (**) represent statistical significant difference with $P \leq 0.05$ and $P \leq 0.01$ comparing with *C. jejuni* RM1221, respectively.

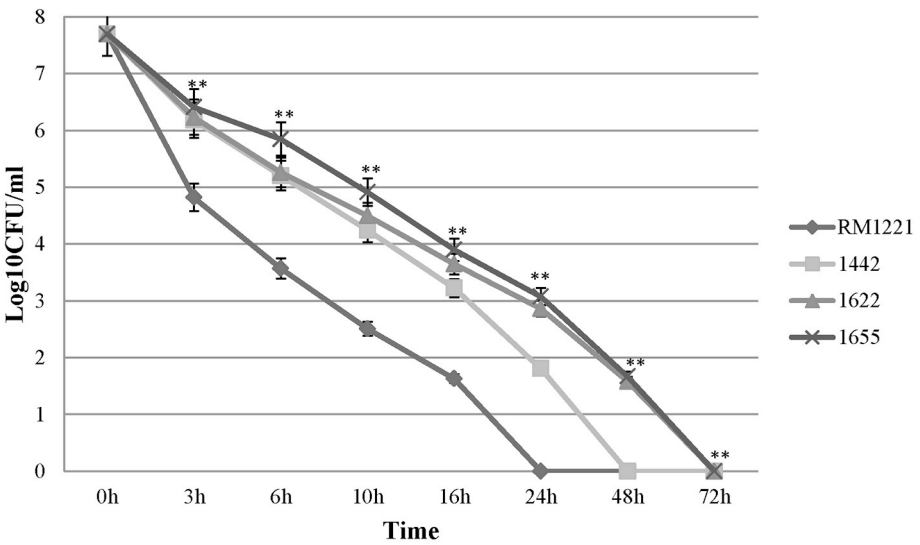

FIGURE 5 | Intra-macrophage survival assay of three selected *C. jejuni* strains in macrophage RAW264.7 cell. The Y-axis is the mean of log10 CFU/mL of each strain in the cell. The asterisk (*) and (**) represent statistical significant difference with $P \leq 0.05$ and $P \leq 0.01$ comparing with *C. jejuni* RM1221, respectively.

which mediated resistance to aminoglycosides. No mutation wad found in the regulators (CmeR and CosR) of CmeABC efflux pumps.

Virulence Associated Genes in *C. jejuni* 1655

A large number of virulence associated genes were found in the genome of 1655 (**Table 4**). Among the selected genes involved in flagella synthesis and assembly (*flaA, flaC, flgA, flgB, flhB, fliM, and fliY*), the *flaC* was found in the genome of 1655 and 11168. Two genes (*ciaB* and *nlpC*) associated with invasion and three genes (*cadF, galE,* and *peb2*) related to adhesion were present in 1655 but not in RM1221. Compared to the genes of CPS biosynthesis system in RM1221, the genome of 1655 contained a restriction modification enzyme, sugar nucleotidyltransferase, capsule polysaccharide protein, and alginate O-acetyltransferase. The genome of 1655 also contained some genes (*cfrA* and ExbB-ExbD-TonB) involved

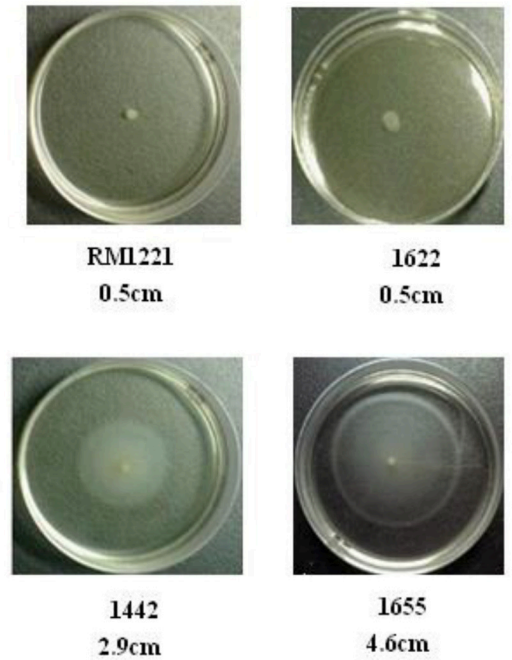

FIGURE 6 | Motility of the three selected *C. jejuni* strains on 0.4% MH plate. The motility is positively corrleated to the diameter of growth ring of each strain. The diameter of spotted colonies was 0.5 cm.

TABLE 3 | Genomic information of target bacteria 1655 and reference strains.

Genomic contents	1655	NCTC11168	RM1221	81–176
Refseq number		NC_002163.1	NC_003912.7	NC_008787
Homology (%)	–	88.78	90	90.44
Genome size (bp)	1720061	1641481	1777831	1616554
G+C (%)	31.36	30.5	30.31	30.6
Predicted coding region	1733	–	1898	1770
CDS	1732	1668	1783	1680
Proteins	–	1572	1783	1449
Pseudo gene	323	40	58	35
rRNA	9	9	9	9
tRNA	40	43	44	44
Other RNA	326	4	4	2

in iron uptake system, while RM1221 lacked TonB and 81176 lacked *cfrA* and TonB13. The IS606 transposase, ISHa1675 transposase B and prophage Lp2 protein 6 were also found in the genome of 1655. ISHa1675 transposase B was not found in the genome of three reference strains. The sequence of prophage Lp2 protein in *C. jejuni* 1655 had 99% homology to that in 81–176. The pVir plasmid which previously found in 81–176, was not found in 1655. Three predicted genes of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated system were found in the genome of 1655. They may encode Csn1, Cas1, and Cas2 proteins respectively.

DISCUSSION

Compared to the genomes of reference strains (NCTC11168, 81–176, RM1221), the genome of 1655 contained some gene inversion, disordered distribution, deletions and rearrangements. The genome difference may be attributed to their different biological characteristics and different evolutional environment. NCTC11168 was originally isolated from a case of human enteritis (Parkhill et al., 2000). 81–176 was originally isolated from a diarrheal outbreak associated with raw milk consumption and exhibited high invasion and high pathogenicity for monkeys and humans (Korlath et al., 1985; Black et al., 1988; Tribble et al., 2010). *C. jejuni* RM1221 (ATCC BAA-1032) was isolated from chicken and showed low virulence (Fouts et al., 2005). 1655 was a multidrug resistant strain isolated from chicken fecal samples that exhibited relative higher virulence, as evidence by its higher pathogenicity to chickens, colonization of the chicken intestinal tract, cytotoxin production, biofilm formation, adhesion/invasion/intracellular survivability to macrophage cell and higher motility.

1655 had multidrug resistance to fluoroquinolones, macrolides, tetracyclines and aminoglycosides. The single T86I mutation in *gyrA* gene contributed to its high-level resistance to FQs (Luangtongkum et al., 2009). High level macrolide resistance in *C. jejuni* 1655 was likely due to A2075G mutation in 23S rRNA (Hao et al., 2009; Luangtongkum et al., 2009; Hao et al., 2010). Although there were some other mutations and deletions in *gyrB,* 23s rRNA genes and ribosomal L4/L22, the function of these mutations on antimicrobial resistance was not evident. The high-level resistance to tetracycline was mediated by *tetO* gene which was located in pTet plasmid (Avrain et al., 2004). The pTet plasmid in 1655 was identical to the one found in 81–176 (Bacon et al., 2002). As a mobile gene element, pTet plasmid may act as a vehicle to pick up and spread multiple antibiotic resistance genes and virulence genes in *C. jejuni* (chen et al., 2013). The *aadE* and *aphA-3* genes in 1655 could explain its resistance to amikacin and gentamicin (Gibreel et al., 2004; Iovine, 2013).

Generally it has been through that multidrug resistance is often associated with higher fitness costs or less virulence (Luangtongkum et al., 2009). However, our study suggested that the multidrug resistant 1655 had higher virulence than the reference strain RM1221 that is not multidrug resistant. Previous studies indicated that T86I mutation in *gyrA* gene could affect DNA supercoiling to regulate the expression of genes associated with bacterial fitness, and therefore enhance the fitness of fluoroquinolone-resistant *Campylobacter* in chicken host and increase the severity and duration of *Campylobacterosis* (Nelson et al., 2004; Luo et al., 2005; Helms et al., 2005; Evans et al., 2009; Han et al., 2012). However, mutations in 23S rRNA could lead to fitness cost and reduced virulence of

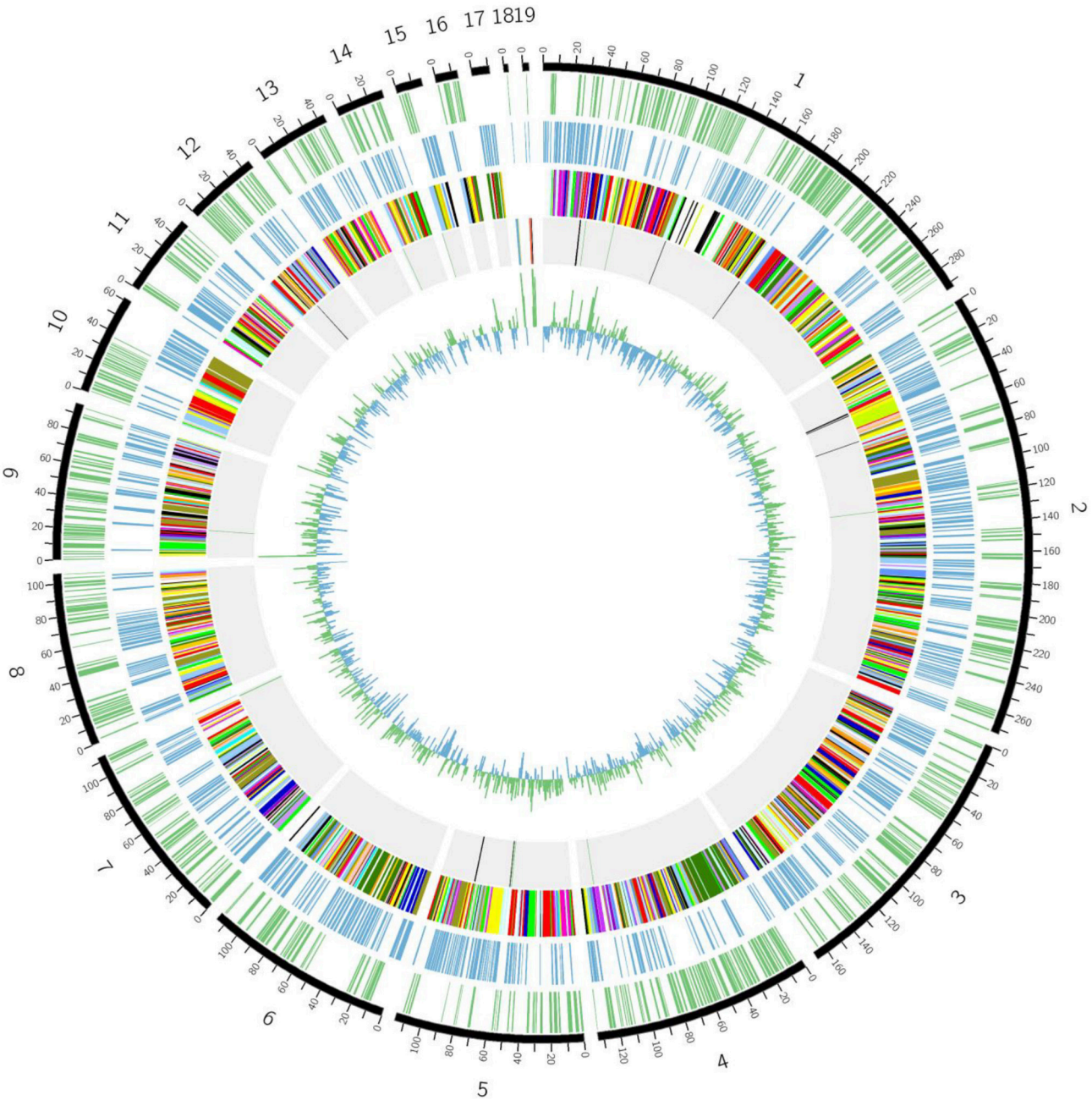

FIGURE 7 | Schematic circular genome of *C. jejuni* 1655 strain. From outside to inside, there were five rings. The first circle shows the positive strand genes. The second circle shows the negative strand genes. The third circle were COG functional annotations (chr1-RNA processing and modification; chr2-Chromatin structure and dynamics; chr3-Energy production and conversion; chr4-Cell cycle control, cell division, chromosome partitioning; chr5-Amino acid transport and metabolism; chr6-Nucleotide transport and metabolism; chr7-Carbohydrate transport and metabolism; chr8-Coenzyme transport and metabolism; chr9-Lipid transport and metabolism; chr10-Translation, ribosomal structure and biogenesis; chr11-Transcription; chr12-Replication, recombination and repair; chr13- Cell wall/membrane/envelope biogenesis; chr14-Cell motility; chr15-Posttranslational modification, protein turnover, chaperones; chr16-Inorganic ion transport and metabolism; chr17-Secondary metabolites biosynthesis, transport and catabolism; chr18-General function prediction only; chr19-Signal transduction mechanisms). The fourth circle are rRNA and tRNA (red-16s rRNA, blue-23S rRNA, yellow-5S rRNA, black-positive chain tRNA, green-negative chain tRNA). The fifth circle is the GC contents.

macrolide resistant *Campylobacter* (Hao et al., 2009; Almofti et al., 2011a,b; Hao et al., 2013). Some of the clinical investigation data indicated the positive correlation between virulence and resistance, but others indicated the negative correlation (Bagger-Skjøt et al., 2007; McGowan-Spicer et al., 2008). For example, two recent studies showed that prevalence

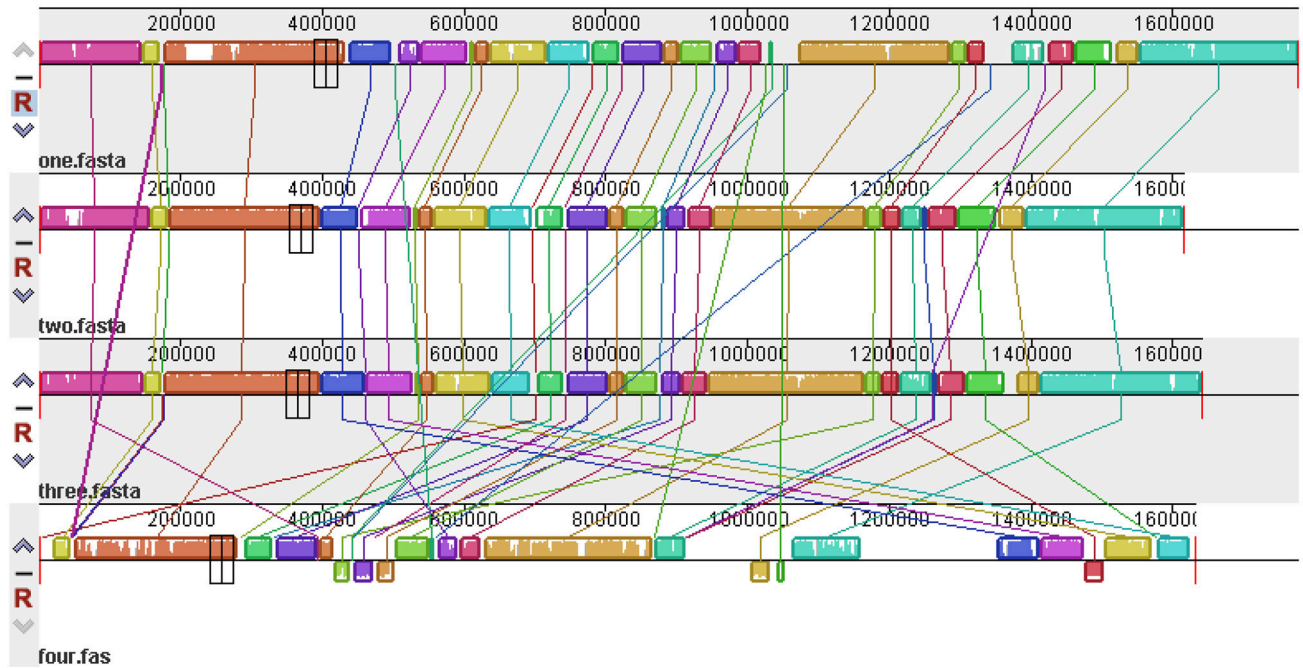

FIGURE 8 | Global multiple alignment of *C. jejuni* genomes. From top to bottom are the genome structures of *C. jejuni* RM1221, 81–176, NCTC11168, and 1655, respectively. The color region and white region represented high and low similarity respectively. Above axis was the positive strand, below was the negative strand.

of resistance to both erythromycin and ciprofloxacin was higher in isolates harboring *ciaB* and some virulence genes (e.g., *cdtA* and *dnaJ*) were associated with antimicrobial-resistant Campylobacter strains (Ghunaim et al., 2015; Lapierre et al., 2016). Our study revealed the relationship between multidrug-resistance and virulence of some *C. jejuni* chicken isolates, indicating that complex mechanisms may work together to cause multidrug resistance and the relative higher virulence in *C. jejuni 1655*.

Comparing to the genome of RM1221, 1655 contained some special virulence-associated genes and proteins, including *flaC, ciaB, nlpC, cadF, gelE*, *peb2*, restriction modification enzyme, sugar nucleotidyltransferase, capsule polysaccharide protein, alginate O-acetyltransferase and TonB iron uptake system. The *flaC* has been shown to modulate immune response in the intestinal tract and contribute to bacterial persistence of *C. jejuni* (Faber et al., 2015). The presence of *ciaB*, a *Campylobacter* invasion antigen, may contribute to the high invasion of *C. jejuni* 1655 (Rivera-Amill and Konkel, 1999). The *nlpC* may play a critical role in initial infection process, adherence to host cells (Padhi et al., 2016). The *cadF*, encoding fibronectin protein, may play an important role on the adhesion and colonization of 1655 in chicken (Konkel et al., 1997; Monteville et al., 2003). The *peb2* is a membrane associated protein which may contribute to the increased gastrointestinal virulence of *C. jejuni* (Cordwell et al., 2008). The capsule polysaccharide (CPS), lipooligosaccharide (LOS), and restriction-modification (R/M) systems may enhance the invasion and colonization of 1655 in reservoir hosts and mediate the virulence of 1655 by evading host immune response (Bacon et al., 2001; Suerbaum et al., 2001; Ahmed et al., 2002; Guerry et al., 2002; Klena et al., 2004; Poly et al., 2004; Fouts et al., 2005; Guerry and Szymanski, 2008). The outer membrane protein CfrA and energy transporter system TonB-ExbB-ExbD are associated with iron absorption and iron uptake in gram-negative bacteria (Krewulak and Vogel, 2011). The presence of CfrA and TonB-ExbB-ExbD system in 1655 may contribute to its stronger ability of nutrients uptake and higher intracellular survivability (Andrews et al., 2003; Hofreuter et al., 2006; Krewulak and Vogel, 2011).

The genes encoding IS606 transposase, ISHa1675 transposase B and prophage Lp2 protein 6 were identified in the genome of 1655, suggesting that 1655 may contain transposons or phages. However, future experiments are needed to determine if these are associated with active mobile genetic elements in 1655. The ISHa1675 transposase B and prophage Lp2 protein 6 were not found in the genome of RM1221 and 11168, indicating that these two genes may play special role in the physiological characteristic of *C. jejuni* 1655.

Three proteins associated with CRISPR-Cas systems (CRISPR-Cas2, CRISPR-Cas1 and a hypothetical protein associated with the CRISPR) were found in 1655. The CRISPR-Cas system provides bacterial defense against foreign nucleic acids derived from bacteriophages or plasmids (Barrangou et al., 2007; Marraffini, 2013). It also plays an important role in gene regulation and bacterial pathogenicity (Mojica et al., 2005;

TABLE 4 | Virulence relative factors in *C. jejuni* 1655 and reference strains.

Virulence genes	1655	1221	11168	81176
Virulence	Relative high	No	Low	Intermediate
pVir Plasmid	−	−	−	+
FLAGELLA SYNTHESIS GENES				
flaA	+	+	+	+
flaC	+	−	+	−
flhB	+	+	+	+
flgB	+	+	+	+
fliM	+	+	+	+
fliY	+	+	+	+
INVASION				
ciaB	+	−	+	−
nlpC	+	−	−	+
ADHESION				
cadF	+	−	+	−
galE	+	−	−	−
Peb1	+	+	+	+
Peb2	+	−	+	−
CHEMOTAXIS				
cheY	+	+	+	+
motB	+	+	+	+
dnaJ	+	+	+	+
CPS BIOSYNTHESIS SYSTEM				
Restriction modification enzyme	+	−	+	−
LPS biosynthesis protein	−	+	−	+
Sugar nucleotidyltransferase (CPS)	+	−	+	+
Capsule polysaccharide protein (KPS)	+	−	+	−
Alginate O-acetyltransferase AlgI	+	−	−	−
IRON UPTAKE SYSTEM				
cfrA	+	+	+	−
TonB123	+	−	+	−
ExbB	+	+	+	+
ExbD	+	+	+	+
TRANSPOSASE AND PHAGES PROTEINS				
IS606 transposase	+	+	+	−
ISHa1675 transposase B	+	−	−	−
Prophage Lp2 protein 6	+	−	−	+
CRISPR-CAS SYSTEM				
CRISPR	*csn1-cas1-cas2*	*csn1-cas1-cas2*	*csn1-cas1-cas2-CRISPR-cstIII*	*cstII*

Louwen et al., 2013; Louwen and van Baarlen, 2013; Sampson and Weiss, 2014). Strains containing CRISPR-Cas system have a stronger ability of biofilm formation and colonization in mouse organs than those strains lack this system (Shimomura et al., 2011). Therefore, the presence of CRISPR-Cas system may contribute to the high virulence and biofilm formation capacity of *C. jejuni* 1655 strain.

CONCLUSION

1655 strain exhibited multidrug resistance to fluoroquinolone, macrolide, tetracycline, and aminoglycoside drugs and relative high *in vitro* and *in vivo* virulence. Comparing to the genome of reference strains (NCTC11168, RM1221, and 81–176), there were large difference in the genome structure and genome content in 1655. The co-emergence of target gene mutations, resistance genes and pTet plasmid could explain its multidrug resistance. The virulence mechanism of *Campylobacter* may be mediated by a variety of virulence factors, including proteins involved in flagella biosynthesis, invasion, adhesion, CPS biosynthesis system, iron uptake system, transposase, phage proteins, and CRISPR-Cas system. Future experiments are needed to find the deeper molecular mechanisms and confirm the function of some important gene elements on multidrug resistance and relative higher virulence.

FINANCIAL DISCLOSURE

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: HH, NR, MD, GC, ZY. Performed the experiments: NR, XK, JL, ZI, HH. Analyzed the data: HH, NR, JH, SF, YW, ZY. Contributed reagents/materials/analysis tools: YW, ZL, MD, YW, ZY. Wrote the paper: HH, NR, JH, SF, ZI, ZY.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01605

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PERSPECTIVE
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Is Quorum Sensing Interference a Viable Alternative to Treat *Pseudomonas aeruginosa* Infections?

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Quorum sensing (QS) coordinates the expression of multiple virulence factors in *Pseudomonas aeruginosa*; hence its inhibition has been postulated as a new alternative to treat its infections. In particular, QS interference approaches claim that they attenuate bacterial virulence without directly decreasing bacterial growth and suggest that *in vivo* the immune system would control the infections. Moreover, since *in vitro* experiments performed in rich medium demonstrate that interfering with QS decreases the production of virulence factors without affecting bacterial growth it was assumed than *in vivo* therapies will minimize the selection of resistant strains. Therefore, the underlying assumptions toward an effective implementation of a successful Quorum sensing interference (QSI) therapy for treating *P. aeruginosa* infections are that (i) QS only exerts important effects in the regulation of virulence genes but it does not affect metabolic processes linked to growth, (ii) the expression of virulence factors is only positively regulated by QS, (iii) inhibition of virulence factors *in vivo* do not affect bacterial growth, (iv) the immune system of the infected patients will be able to get rid of the infections, and (v) the therapy will be effective in the strains that are actively producing the infections. Nevertheless, for QSI in *P. aeruginosa,* substantial experimental evidence against the validity of most of these assumptions has accumulated during the past years, suggesting that a far better understanding of its virulence and its behavior during infections is needed in order to design truly solid QSI therapeutic alternatives to combat this remarkable pathogen.

Keywords: ***Pseudomonas*** **infections, quorum sensing (QS), quorum quenching, resistance, clinical isolates**

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INTRODUCTION

Pseudomonas aeruginosa is a remarkable opportunistic pathogen that infects patients that are immunocompromised, have severe burns, cancer, or AIDS, are intubated and with prosthetic devices, and also those suffering from chronic affections like cystic fibrosis (CF). This bacterium is a major health problem worldwide being responsible of 10% of nosocomial infections (Antunes et al., 2010; Castillo-Juárez et al., 2015), since it is intrinsically resistant to several antimicrobials (Poole, 2011) and able to develop resistance against new ones, has a high biofilm production and produces an arsenal of virulence factors. One of the main mechanisms that controls the production of its virulence factors is quorum sensing (QS) which coordinates the expression of such factors

once bacteria have reached a high population density, thus maximizing their chances to overcome the host defenses and establish the infection.

QS systems are common in bacterial pathogens; among Gram negatives, in addition to *P. aeruginosa, Acinetobacter baumannii* (Bhargava et al., 2010), *Escherichia coli* (Sperandio et al., 2002) Salmonella strains (Choi et al., 2007), and Vibrio strains (Zhu et al., 2002; Yang and Defoirdt, 2015) use them to coordinate their virulence. Hence, QS interference (QSI) or quorum quenching (QQ) is a strategy proposed to inhibit virulence as an alternative to treat the infections of several important bacterial pathogens (Castillo-Juárez et al., 2015).

In this opinion piece I focus in *P. aeruginosa*, one of the most studied organisms regarding QS and QSI, nevertheless what is exposed here may be also applicable to other bacterial pathogens.

P. aeruginosa is one of the more complex known bacterial pathogens; it possesses an ample genome and a high percentage of its genes are devoted to gene regulation (Stover et al., 2000). Regarding QS systems, it has a hierarchical architecture governed by the LasRI module which produces and senses N-3-oxo-dodecanoyl-L-Homoserine lactone and activates the expression of multiple virulence genes such as those producing elastase A and B, pyocyanin, alkaline and protease, and activating a second homoserine lactone (HSL) QS module known as RhlRI, which produces and senses N-butyryl-L-Homoserine lactone. RhlR bound to its autoinducer also activates directly some virulence genes like those encoding rhamnolipids and pyocyanin (Smith and Iglewski, 2003; Jimenez et al., 2012). In addition, *P. aeruginosa* also has a quinolone dependent system known as PQS, which is positively regulated by LasR and negatively by RhlR, forming a complex and intricate network (Jimenez et al., 2012; Lee and Zhang, 2015). In addition to the QS intrinsic components, several regulators such as GacA/GacS, QscR, Vfr, RpoN, and RpoS, influence the expression of QS dependent factors (Lee and Zhang, 2015).

The complex relationship between QS and virulence in *P. aeruginosa* had been recently evidenced by the fact that *lasR* deficient mutants (including clinical strains) growing at slow rates or in the stationary phase, overproduce pyocyanin due a lack of repression of the phenazine genes by RsaL, a negative transcriptional regulator positively controlled by LasR (Cabeen, 2014). Moreover, in *lasR* mutants, the activity of the *rhl* system is only delayed but not abolished, allowing significant production of pyocyanin, rhamnolipids and N-butyryl-L-Homoserine lactone (which are RhlR dependent) and even the production of the LasR dependent QS signals N-3-oxo-dodecanoyl-L-Homoserine lactone and PQS (Dekimpe and Deziel, 2009). These findings suggest that the inhibition of a particular component of the QS network, even the pivotal factor LasR, may be counteracted by the activation of alternative components of the network. Indeed, some recent studies have identified novel QS inhibitors for which RhlR and not LasR as the relevant *in vivo* target (O'Loughlin et al., 2013; Welsh et al., 2015).

In addition, recently it was discovered that an environmental strain (148 isolated from dolphin gastric juice) produces significant amounts of the QS-controlled virulence factors rhamnolipids and pyocyanin. Critically, this strain is virulent to mice, even without having a *lasR* gene and without producing N-3-oxo-dodecanoyl-L-Homoserine lactone. Hence, in this strain, the control of QS dependent virulence factors must be exerted by other regulators (Grosso-Becerra et al., 2014).

QS Interference could be achieved by the attenuation of the QS communication systems via: (i) the disruption of the QS receptors, (ii) the degradation of the autoinducers signals or (iii) the inhibition of the signal synthesis. Accordingly, several QS inhibitors or quorum quenchers (QQ) suitable for *P. aeruginosa* have been developed, under the assumptions that (i) the expression of virulence factors is only positively regulated by QS, (ii) the QQ will only exert significant effects in the regulation of virulence genes but not in metabolic processes linked to growth, hence avoiding or at least decreasing the generation of selective pressure that leads to resistance, (iii) the immune system of the infected patients will be able to get rid of the infections, and (iv) the therapy will be effective against the strains that are actively producing the infections. Nevertheless, substantial evidence against the validity of those assumptions has accumulated over the years.

QUORUM SENSING INFLUENCES METABOLIC PROCESSES AND BACTERIAL GROWTH

Although the role of QS in *P. aeruginosa* virulence has been extensively studied, whether it influences global metabolism and cell growth is less explored. One possible reason for this bias is that at the transcriptomic level, few significant changes in metabolicrelated genes are found when QS proficient and deficient strains are compared or when QS inhibitors are administrated to QS proficient strains (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). Nevertheless, recently Davenport and coworkers found that the metabolome of a *lasI rhlI* double mutant (unable to produce HSL QS autoinducers) and that of its wild-type progenitor have a notorious divergence once the wild-type strain produced the highest levels of autoinducers; remarkably, around one third of all the metabolites identified changed (Davenport et al., 2015). This phenomenon is understandable since the wild-type strain devotes many of its resources to the production of costly virulence factors such as exoproteases, phenazines, and exopolysacharides, while, in contrast, the QS mutants uses the same resources for cell division. In agreement, the QS deficient mutants achieve higher growth yields than QS wild-type strains (Diggle et al., 2007).

Although the possible implications of the metabolic divergence in QS mutants on bacterial physiology and QS interference therapies is still unexplored, Davenport and coworkers demonstrated that important metabolic changes in the membrane metabolism of the wild-type strain are driven by QS upon entering the stationary phase. These modifications include increased fatty acid saturation, chain length, and cyclopropanation, which in turn, promote the generation of robust cell membranes. In contrast, the membranes of QS mutants do not have these modifications and are therefore more susceptible to stress.

In addition to these metabolic changes, several works have demonstrated that in *P. aeruginosa* and other bacteria, QS enhances the stress response (Bjarnsholt et al., 2005; Bhargava et al., 2014; de Oca-Mejia et al., 2015; García-Contreras et al., 2015a); for example, by upregulating antioxidant enzymes such as catalase, superoxide dismutase (Hassett et al., 1999) and NADPH-generating enzymes (García-Contreras et al., 2015a). This is important since during an infection, the immune system attacks bacteria by releasing reactive oxygen species, and at least *in vitro* oxidative stress is able to select functional QS systems as well as QS interference resistant mutants (García-Contreras et al., 2015a).

Moreover, growth of *P. aeruginosa* can be strongly dependent on QS either if adenosine or protein is used as sole carbon sources, since *nuh*, the gene encoding the nucleoside hydrolase, is under tight LasR control (Heurlier et al., 2005) and since the expression of exoproteases is under QS control (Diggle et al., 2007). During infections, adenosine utilization (Patel et al., 2007; Sheng et al., 2012) as well as the degradation of host proteins (Wretlind and Pavlovskis, 1983; Laarman et al., 2013) are important for bacterial virulence and survival.

All the above summarized facts demonstrate that QS is an important regulator of bacterial metabolism and physiology and suggest that QS interference will have the direct effect of decreasing bacterial growth and viability *in vivo*. In agreement, several *in vivo* infection studies have shown that when a QS interference therapy is able to increase animal survival (and decrease damage to the host), there is a significant decrease in bacterial counts in the infection sites (Wu et al., 2004; Christensen et al., 2007; Defoirdt et al., 2010; Jakobsen et al., 2011). The decrease in bacterial viability due QS interference may be an important potential source of *in vivo* selective pressure for the selection of bacterial resistance (García-Contreras et al., 2016).

POTENTIAL INCREASE IN VIRULENCE UPON QUORUM SENSING INTERFERENCE

Although the production of multiple virulence factors in *P. aeruginosa* is positively regulated by QS, the virulence phenotype displayed by this organism is a complex combinatorial phenomenon that cannot be easily predicted with the identification of the presence or absence of a set of specific genes (Lee et al., 2006; Grosso-Becerra et al., 2014). In agreement, several studies had shown that clinical strains display a wide variety levels of virulence (Fenner et al., 2006; Lee et al., 2006; Garcia-Contreras et al., 2015b) and that environmental strains often conserve high virulence (Grosso-Becerra et al., 2014).

An overlooked fact about the role of QS in *P. aeruginosa* virulence was discovered in 2005 by Bleves and coworkers, who that found that in contrast to several other virulence traits, the expression of the type III secretion system (TTSS) in *P. aeruginosa* is negatively regulated by QS, specifically by RhlR and PqsR (Bleves et al., 2005; Kong et al., 2009). An important factor in such negative regulation of virulence genes by QS is that it requires low calcium levels, and hence several transcriptomics studies done in culture medium such as LB with relative high calcium levels fail to show this relationship. Since the TTSS is an important determinant of virulence in several animal infection models including pneumonia, peritonitis, bacteremia, burn infections, and keratitis (Hauser, 2009), the possibility that QS interfering therapies may be activating this system and therefore promoting virulence should not be ignored. Moreover, *P. aeruginosa* has five of the six known types of secretion systems present in Gram negative bacteria (all but type IV) and some of these systems, like type VI, are present in several copies in its genome (Bleves et al., 2010). The QS influence on the expression of these secretion systems is as of yet unknown, with the exceptions of the TTSS and the second type VI secretion system that are negatively regulated by QS (Sana et al., 2012).

More striking is the fact that QS interference using azithromycin selects the wild-type virulent phenotype against the less virulent *lasR* mutants in intubated patients colonized by *P. aeruginosa* (Kohler et al., 2010). This surprising fact is understandable since *lasR* mutants often appear and are selected in infections since they act as phenotypic cheaters that utilize the public goods such as exoproteases and siderophores produced by the cooperative wild-type individuals (Diggle et al., 2007; Sandoz et al., 2007). Hence, inhibiting QS removes the advantage of the *lasR* mutants and thereby selects the wild-type, thus a detrimental effect of QS interference in the long run could be to increase the prevalence of virulent genotypes in the nosocomial environment (Kohler et al., 2010).

Another key aspect of current QS inhibitors like furanones is that, depending on their concentration, they can activate rather than inhibit QS (Martinelli et al., 2004). Furthermore, related QS inhibitors such as synthetic HSLs can activate rather than inhibit some virulence factors (Welsh et al., 2015).

LIMITATIONS OF THE CURRENT ANIMAL INFECTION MODELS FOR THE STUDY OF QUORUM SENSING INTERFERENCE

To date, several animal infection models have demonstrated that QS deficient mutants are much less virulent than their QS proficient parental strains; accordingly, QS interference promotes an increase in host survival and a decrease in damage and bacterial counts (Castillo-Juárez et al., 2015). The infection models used include arthropods like *Galleria mellonela*, nematodes such as *Caenorhabditis elegans*, fruit fly, and zebrafish which are valuable and informative but that do not accurately reflect human physiology. Moreover, when mice are used as a model, immune competent individuals are evaluated; hence, the fact that *P. aeruginosa* is a strict opportunistic pathogen that does not attack individuals with competent immune systems is overlooked, and so it is not clear if the immune systems of immunosuppressed patients will be able to clear the bacteria from infections upon QS interference treatments.

In addition to attacking immunosupressed individuals, *P. aeruginosa* is the major cause of death of CF patients; accordingly, there are several mice models that incorporate

different mutations in the CF transmembrane conductance regulator (CTFR) protein and that are able to mimic some of the characteristics of the disease in humans. However, CF is a very complex disease and there are more than one thousand reported mutations in the *cftr* gene associated with the disease; hence, developing a murine model that closely resembles the human disease is challenging (Guilbault et al., 2007).

Most of the current models are suitable to study acute infections and only a few like the one developed in 2005 by Hoffmann and coworkers are optimized to simulate chronic CF infections (Hoffmann et al., 2005). Importantly, this model has been used to demonstrate that QS interference with azithromycin inhibits alginate production of the mucoid NH57388A strain *in vivo*, attenuating the damage produced to the host, but unfortunately it was not able to significantly increase mouse survival (Hoffmann et al., 2007).

Clinical studies have demonstrated that AZM treatment improves lung function in CF patients (Saiman et al., 2003); however, besides the QSI effect, azithromycin has bactericidal and anti-inflammatory effects; hence, the improvement of both mice and patients is likely due a combination of these effects rather than an exclusive consequence of the QSI properties of azithromycin. Therefore, testing of the effect of more specific QS interference molecules in these kind of models is needed in order to elucidate the potential of these therapies for CF patients.

Another kind of mouse model that more closely resembles the situation observed in humans is the thermally-induced injury model, which consists of producing a burn of second or third degree on the dorsal side of the mouse using hot water and subsequent inoculation of the burn. The utilization of this model has confirmed that QS-deficient mutants such as *lasR*, *lasI*, *rhlI*, *lasI rhlI*, and *pqsA* (Rumbaugh et al., 1999a,b; Lesic et al., 2007), have less virulence than the parental strains. Moreover, it has been used to test the effect of the halogenated anthranilic acid analogs 6FABA, 6CABA, and 4CABA which are inhibitors of the synthesis of quinolone QS signals. These compounds, at the doses administrated, decrease mouse mortality significantly. Nevertheless, they were tested at a single dose (Lesic et al., 2007), so it is unknown if they exert dose response effects. This is not trivial since as mentioned before, QS inhibitors may act as QS activators depending on the used doses (Martinelli et al., 2004).

INSENSIBILITY OF SOME CLINICAL STRAINS TO CURRENT QUORUM QUENCHERS

Before 2010 it was assumed than QS interference will be impervious or at least less susceptible to promote bacterial resistance than conventional antibiotic therapies (Defoirdt et al., 2010). In 2011, it was demonstrated that *P. aeruginosa* acquires resistance easily against the canonical HSL-dependant quorum quencher furanone C-30, by activating the multidrug efflux pump MexAB-OprM (Maeda et al., 2012). The activation of this pump is mediated by mutations disrupting the transcriptional repressors MexR and NalC, and such mutations are common in clinical isolates, presumably since they are selected by intense antibiotic treatments (Tomas et al., 2010). As expected, these clinical isolates are resistant against C-30 (Maeda et al., 2012).

Recent studies have demonstrated that C-30 resistance is common in multidrug resistant strains. Importantly, resistance against other QQ compounds like 5-fluorouracil is also present in some clinical isolates and some antibiotic sensitive strains are also resistant to C-30 by a decrease in the compound uptake. More strikingly, some of these strains produce higher amounts of the virulence factors in the presence of the furanone than in basal conditions (García-Contreras et al., 2013a,b; Garcia-Contreras et al., 2015b).

This suggests that if eventually QSI therapies are implemented in the clinic, they likely would not be effective against all the strains present in infections (García-Contreras et al., 2013a; Kalia et al., 2013), and that crucially they may fail to inhibit those strains with active antibiotic efflux pumps. Indeed, it was recently proposed that bacteria resistant to several QS inhibitors may be selected by QSI treatments (Koul et al., 2016).

PERSPECTIVES

Since recent evidence has demonstrated that QS is linked to basal metabolism and growth, it will be important to evaluate QS interference and its effect in bacteria growing in medium that more closely simulates the composition of the environment during infection (Palmer et al., 2007; Garcia-Contreras et al., 2015b), with attention on key metabolites that influence virulence and QS such as iron (Sokol and Woods, 1984; Mittal et al., 2008; Hazan et al., 2010), calcium (Sarkisova et al., 2005), phosphate (Zaborin et al., 2009), and adenosine (Patel et al., 2007; Sheng et al., 2012). Further, metabolomic and proteomic studies may shed light about the role of QS in bacterial physiology under *in vivo*-like conditions. In addition, the utilization of new technologies like microfluidics such as cell culture chips (organ-on-a-chip) would enable the interrogation of the roles of signaling, social cheating, mass transfer, and spatial organization in well-defined geometries, which is virtually impossible to asses at this moment using animal models.

Importantly, additional research about the ways *P. aeruginosa* may achieve resistance against QQ is needed in order to generate effective combination therapies less prone to the selection of resistance. One possibility is to exploit the fact that QS interference renders *P. aeruginosa* more sensitive to stress, including antibiotics and the effects of the immune system; hence, it is attractive to test if the combination of QS interference, antibiotic therapies and immunotherapy to improve the outcome of the therapies.

Another area of research that should be encouraged is the role of QS independent virulence factors and those negatively regulated by QS such as TTSS in infections and the effect of QS interference on these virulence determinants. Also, study of QS networks and the-virulence of environmental and clinical strains is needed since current evidence indicates these are highly variable (Grosso-Becerra et al., 2014; Garcia-Contreras et al.,

2015b) and that a specific QQ compound will not always be effective against all clinical strains. Also, more effort should be devoted to the study of the implementation of these therapies, since most of them are able to attenuate infections when the QQ compound is administrated shortly before the inoculation (having possible prophylactic effects), but the value of such therapies as a possible cure for an established infection should be better addressed.

Taking the available evidence together, it seems QS interference may be a valuable tool to combat *P. aeruginosa* infections; however, using QS inhibition as the sole therapy may not be an efficient strategy due several potential drawbacks. Hence, before QSI applications are used in the clinic, it is advisable to improve our current understanding of *P. aeruginosa* virulence with an emphasis on clinical strains and also on further defining the roles of QS in the physiology of this remarkable complex pathogen.

AUTHOR CONTRIBUTIONS

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

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PA3297 Counteracts Antimicrobial Effects of Azithromycin in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa causes acute and chronic infections in human. Its increasing resistance to antibiotics requires alternative treatments that are more effective than available strategies. Among the alternatives is the unconventional usage of conventional antibiotics, of which the macrolide antibiotic azithromycin (AZM) provides a paradigmatic example. AZM therapy is associated with a small but consistent improvement in respiratory function of cystic fibrosis patients suffering from chronic *P. aeruginosa* infection. Besides immunomodulating activities, AZM represses bacterial genes involved in virulence, quorum sensing, biofilm formation, and motility, all of which are due to stalling of ribosome and depletion of cellular tRNA pool. However, how *P. aeruginosa* responds to and counteracts the effects of AZM remain elusive. Here, we found that deficiency of PA3297, a gene encoding a DEAH-box helicase, intensified AZM-mediated bacterial killing, suppression of pyocyanin production and swarming motility, and hypersusceptibility to hydrogen peroxide. We demonstrated that expression of PA3297 is induced by the interaction between AZM and ribosome. Importantly, mutation of PA3297 resulted in elevated levels of unprocessed 23S-5S rRNA in the presence of AZM, which might lead to increased susceptibility to AZM-mediated effects. Our results revealed one of the bacterial responses in counteracting the detrimental effects of AZM.

Keywords: RNA helicase, antibiotic resistance, azithromycin, rRNA processing, *Pseudomonas aeruginosa*

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INTRODUCTION

Pseudomonas aeruginosa is a versatile Gram-negative pathogenic bacterium, that can cause various infections in human (de Bentzmann and Plésiat, 2011; Campa et al., 2012). During infection, *P. aeruginosa* produces multiple virulence factors to facilitate colonization (Sadikot et al., 2005; Kipnis et al., 2006; Hauser, 2009; Liu et al., 2015). Meanwhile, its highly intrinsic antibiotic resistance and biofilm forming ability greatly hinder the eradication of this pathogen (Høiby et al., 2005). In patients suffering from cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), *P. aeruginosa* caused chronic respiratory infections are responsible for most of the morbidity and mortality (Rabin et al., 2004; Rada and Leto, 2013). Intensive antibiotic treatment has been used to maintain the lung function and extend lifespan of the patients (Doring et al., 2000). However, the increasing antibiotic resistance has been compromising clinical efficacy of traditional antibiotics. Thus, alternatives or unconventional usage of the antibiotics are urgently needed (Breidenstein et al., 2011; Poole, 2011; Imperi et al., 2014).

The macrolide antibiotic azithromycin (AZM) provides a paradigmatic example of an unconventional antibacterial drug for *P. aeruginosa* treatment. Although, *P. aeruginosa* is highly resistance to macrolides owning to its low outer membrane permeability and the resistance-nodulation-cell division (RND) systems, AZM treatment benefits patients suffering from both intermittent and chronic *P. aeruginosa* infections (Saiman et al., 2003; Lister et al., 2009; Blasi et al., 2010; Steel et al., 2012; Aminov, 2013; Morita et al., 2013). AZM has been shown to have immunomodulatory activity, which attenuates the inflammatory response and promotes macrophage phagocytic activity (Legssyer et al., 2006; Steinkamp et al., 2008; Tsai et al., 2009). Furthermore, AZM exhibits bactericidal effect on stationary growth phase *P. aeruginosa* cells (Lovmar et al., 2004, 2009; Imamura et al., 2005; Köhler et al., 2007; Starosta et al., 2010; Gödeke et al., 2013). And sub-inhibitory concentrations of AZM suppress biofilm formation, motility, and production of multiple virulence factors, including proteases, pyocyanin, exotoxin A, phospholipase C (PLC), exopolysaccharides, and other quorum-sensing (QS) regulated genes in *P. aeruginosa* (Molinari et al., 1992, 1993; Tateda et al., 2001; Favre-Bonté et al., 2003; Gillis and Iglewski, 2004). The AZM-mediated killing of stationary-phase bacterial cells and reduced expression of QS-regulated virulence factors require interaction between AZM and ribosome (Köhler et al., 2007). AZM binds in the nascent peptide exit tunnel (NPET), resulting in ribosome stalling and depletion of the intracellular pools of aminoacyl-tRNAs (Lovmar et al., 2004, 2009; Köhler et al., 2007; Starosta et al., 2010; Gödeke et al., 2013). The effects of AZM on *P. aeruginosa* can be counteracted by over expression of ErmBP or a peptidyl-tRNA hydrolase, which blocks the interaction between AZM and ribosome by modifying the 23S rRNA or increases the intracellular aminoacyl-tRNA level, respectively (Köhler et al., 2007; Gödeke et al., 2013). However, how *P. aeruginosa* response to AZM treatment remains unclear. Understanding the mechanisms that *P. aeruginosa* uses to counteract AZM treatment may provide clues to enhance AZM-mediated virulence inhibitory and bacterial killing effects.

A large RNA helicase family named DExD/H box helicases are characterized by a conserved DExD/H box sequence (Cordin et al., 2006; Linder and Jankowsky, 2011), and play crucial roles in rRNA processing, translation initiation, and mRNA decay (Iost et al., 2013; Linder and Fuller-Pace, 2013). In addition, the DExD/H box helicases have been shown to participate in bacterial responses to various stresses, such as cold shock, pH, osmotic, and oxidative stresses (Owttrim, 2013). And several DEAD family RNA helicases, which belong to a specific subfamily of DExD/H box helicases, have been shown to regulate virulence factors in *Escherichia coli*, *Borrelia burgdorferi*, *Staphylococcus aureus*, *Listeria monocytogenes,* and *P. aeruginosa* (Koo et al., 2004; Salman-Dilgimen et al., 2011, 2013; Oun et al., 2013; Bareclev et al., 2014; Intile et al., 2015). The pleiotropic functions of DExD/H box family RNA helicases intrigued us to suspect that they might be involved in the bacterial response to AZM treatment. In this study, we found that deficiency in a DEAH box helicase, PA3297, renders *P. aeruginosa* more susceptible to the killing and virulence suppression by AZM. Our results suggest that the expression of PA3297 was up regulated in the presence of AZM, which might promote 23S rRNA maturation to counteract the inhibitory effect of AZM on protein elongation.

MATERIALS AND METHODS

Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in **Table 1** (Simon et al., 1983; Hoang et al., 1998; Choi and Schweizer, 2006; Liberati et al., 2006). The *E. coli* strains DH5α, S17-1 and *P. aeruginosa* strains were routinely cultured in Luria-Bertani (LB) broth at 37°C. Antibiotics were used at the following concentrations: for *E. coli*, ampicillin 100 μg/ml, tetracycline 10 μg/ml, and gentamicin 10 μg/ml; for *P. aeruginosa*, carbenicillin 150 μg/ml, tetracycline 50 μg/ml, and gentamicin 50 μg/ml.

DNA Methods

DNA manipulations were performed according to standard protocols or following manufacturers' instructions (Hoang et al., 1998; Zheng et al., 2004; Choi and Schweizer, 2006). The pEX18Tc::ΔPA3297 (pTH1501) was constructed by cloning the 1002-bp upstream and 964-bp downstream fragments of PA3297 coding region into the KpnI-HindIII sites of plasmid pEX18Tc. The fragments were amplified from the PA14 chromosome with primers PA14-UPA3297-FF, PA14-UPA3297-FR, PA14-DPA3297-FF, and PA14-DPA3297-FR (**Table 2**), respectively. Deletion of the PA3297 gene was confirmed by PCR with primers PA14-PA3297-FF and PA14-PA3297-FR (**Table 2**). For the complementation of PA3297, the PA3297 gene was amplified from the PA14 chromosome by PCR with the primers PA14-PA3297-FF and PA14-PA3297-FR (**Table 2**). The PCR product was ligated into the EcoRI- SacI sites of pUC18t-mini-Tn7T-Gm, resulting in pTH1502. The plasmid was introduced into the ΔPA3297 mutant by electroporation, along with the helper plasmid pTNS3 (Choi and Schweizer, 2006). Insertion of the PA3297 gene into the chromosome was confirmed by PCR with primers P_{Tn7R} and $P_{glmS-down}$ (**Table 2**; Choi and Schweizer, 2006). The site-directed mutagenesis was performed as previously described (Zheng et al., 2004). The mutation sites were chosen based on the conserved critical residues of other bacterial DExD-box proteins (Koo et al., 2004; Cordin et al., 2006). Briefly, PCR amplification was performed with pTH1502 as template and with primers listed in **Table 2**, for K101A, D192A, and SAT224-226AAA mutations, respectively. The PCR products were treated by DpnI for 3 h at 37°C and purified before transformation. The correctly mutated clones were identified by DNA sequencing.

Assay for Pyocyanin Production

The pyocyanin concentration was determined as described previously (Essar et al., 1990). Briefly, 1 ml supernatant from each 24-h-old bacteria culture grown in the absence or presence of AZM was extracted with 0.5 ml of chloroform. Then, 0.4 ml solution from the lower organic phase was re-extracted into

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or origin
***E. coli* strains**		
DH5α	F^-, φ80d*lacZ*ΔM15, Δ*(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17*(r_k^-,m_k^+), *phoA, supE44,* λ^-, *thi1, gyrA96, relA1*	TransGen
S17-1	*thi pro hsdR recA* Tra^+	Simon et al., 1983
***P. aeruginosa* strains**		
PA14	Wild type *Pseudomonas aeruginosa* strain PA14	Liberati et al., 2006
PA0426::Tn	PA14 with a transposon inserted at PA0426	Liberati et al., 2006
PA0455::Tn	PA14 with a transposon inserted at PA0455	Liberati et al., 2006
PA2840::Tn	PA14 with a transposon inserted at PA2840	Liberati et al., 2006
PA3002::Tn	PA14 with a transposon inserted at PA3002	Liberati et al., 2006
PA3272::Tn	PA14 with a transposon inserted at PA3272	Liberati et al., 2006
PA3297::Tn	PA14 with a transposon inserted at PA3297	Liberati et al., 2006
PA3308::Tn	PA14 with a transposon inserted at PA3308	Liberati et al., 2006
PA3861::Tn	PA14 with a transposon inserted at PA3861	Liberati et al., 2006
PA3950::Tn	PA14 with a transposon inserted at PA3950	Liberati et al., 2006
ΔPA3297	PA14 with PA3297 in frame deletion	This study
ΔPA3297/att7::PA3297	PA14 ΔPA3297 with insertion of a single copy of PA3297 driven by its own promoter at attTn7 sites	This study
ΔPA3297/att7::PA3297 K101A	PA14 ΔPA3297 complemented with a single copy of PA3297 acquired a lysine101 mutation to alanine	This study
ΔPA3297/att7::PA3297 D192A	PA14 ΔPA3297 complemented with a single copy of PA3297 acquired an aspartate192 mutation to alanine	This study
ΔPA3297/att7::PA3297SAT224AAA	PA14 ΔPA3297 complemented with a single copy of PA3297 acquired a serine224 and threonine226 mutation to alanines	This study
Plasmids		
pEX18Tc	Broad-host-range gene replacement vector; *sacB* TET^r	Hoang et al., 1998
pUC18t-mini-Tn7T-Gm	For gene insertion in chromosome; GEN^r	Choi and Schweizer, 2006
pTNS3	Helper plasmid	Choi and Schweizer, 2006
pFLP2	Source of Flp recombinase; *sacB*, AMP^r/CAR^r	Hoang et al., 1998
pTH1501	pEX18Tc::ΔPA3297; TET^r	This study
pTH1502	PA3297 gene of PA14 on pUC18T-Mini-Tn7T-Gm with its own promoter; GEN^r	This study

[a]*AMP^r, ampicillin resistant; TET^r, tetracycline resistant; GEN^r, gentamicin resistance; CAR^r, carbenicillin resistant.*

0.3 ml of 0.2 N HCl to give a pink solution, whose absorbance was measured at 520 nm. Concentrations of pyocyanin (mg/ml) were calculated by multiplying the OD_{520} by 32.01 (Kurachi, 1958).

Antibiotic Susceptibility Assay

Minimum inhibitory concentrations (MICs) of *P. aeruginosa* to antimicrobial agents were determined by serial twofold broth dilution in LB medium, as described previously (Jo et al., 2003). MICs were recorded as the lowest concentration of antibiotic inhibiting visible growth after 24 h of incubation at 37°C.

Stationary-Phase Bacterial Cell Killing Assay

The killing assay was performed as described previously (Köhler et al., 2007). Briefly, bacteria were inoculated in LB medium and grown for 16 h at 37°C. The culture of each strain was diluted to an OD_{600} of 0.05 and cultured at 37°C. After reaching stationary phase (OD_{600}, 3.0), indicated concentrations of AZM were added to the 2-ml aliquots of the cultures. Then the bacteria were cultured for 20–22 h at 37°C. The viable bacterial numbers were determined by serial dilution and plating on drug-free LB agar plates. The survival rate of each strain was calculated as live bacterial number in AZM treated sample divided by the bacterial number of the corresponding untreated sample.

Biofilm Tolerance to AZM

The biofilm resistance was measured as previously described with minor modifications (Bjerkan et al., 2009; Billings et al., 2013; Liao et al., 2013). Briefly, overnight bacterial cultures were diluted to an OD_{600} of 0.025. 150 μl of the bacteria were incubated in each well of a 96-well plate at 37°C without agitation (Brencic et al., 2009). After 24 h, the planktonic bacteria were discarded by aspiration. Then, the biofilms were treated with 150 μl LB medium containing indicated concentrations of AZM for 2 h. The medium in each well was replaced with fresh LB medium, and subjected to sonication at a frequency of 40 kHz, with a power output of 300 W, at 37°C for 5 min. The live bacteria were enumerated by serial dilution and plating.

Growth Assay

Overnight culture of each strain was diluted into fresh LB (150 μl) to an OD_{600} of 0.05 in each well of a 96-well plate without or

TABLE 2 | Primers used in this study.

Primers[a]	Nucleotide sequence (5′→3′)[b]
Cloning of upstream and downstream fragments for PA3297 deletion	
PA14-UPA3297-FF	GAAAGCGGTACCGAAGTAAGTCCGCCGTTGCC (Kpn I)
PA14-UPA3297-FR	CAGCTTTCTAGAGGTGCTGTCGTCGCTCTGGT (Xba I)
PA14-DPA3297-FF	TTGCAGTCTAGACGCTGGATGCTGGAGGAGTA (Xba I)
PA14-DPA3297-FR	CGCCGGAAGCTTCACCGAGCAGTGGCTGAAGAC (Hind III)
Cloning of gene PA3297 for complementation	
PA14-PA3297-FF	TGAAGAGAATTCGCCAGAAGTAAGTCCGCCGTTGCC (EcoR I)
PA14-PA3297-FR	CACCGGGAGCTCCGACCAGACCGACCTGTTCTTCA CCAT (Sac I)
P_{Tn7R}	CACAGCATAACTGGACTGATTTC
$P_{glmS-down}$	GCACATCGGCGACGTGCTCTC
Primers used for site-directed mutagenesis of PA3297	
PA3297-K101A-FF	GCGAGACCGGCTCGGGCGCGACCACCCAG
PA3297-K101A-FR	TCGCGCCCGAGCCGGTCTCGCCGGCGATC
PA3297-D192A -FF	TACGACACGCTGATCGTCGCCGAAGCCCAC
PA3297-D192A -FR	CGGCGACGATCAGCGTGTCGTAGCGCTCCAG
PA3297-SAT224-226AAA-FF	GCTGATCATCACCGCGGCGGCCATCGACCTGGAG
PA3297-SAT224-226AAA-FR	TGGCCGCCGCGGTGATGATCAGCTTCAGGTCC
RT-qPCR primers for gene expression measurements	
23S-R-FF	AAAGATAACCGCTGAAAG
23S-R-FR	CTATCAACGTCGTAGTCT
5S-R-FF	CGAACTCAGAAGTGAAAC
5S-R-FR	CTTGACGATGACCTACTC
23S-5S-R -FF	GTACTAATTGCCCGTGAG
23S-5S -R-FR	GTTCCAACGCTCTATGAT

[a]*The direction of the primer is indicated at the end of the primer designation as follows: FF for forward and FR for reverse.* [b]*The solid underlines are the sites of listed restriction enzymes; the dotted underlines are the sites for mutagenesis, with all the residues changed into alanine.*

with different concentrations of AZM. The plate was incubated at 37°C with constant agitation (Lau et al., 2012; Guénard et al., 2014). The bacterial growth was monitored by measuring the OD_{600} every 30 min for 12 h by a Varioskan Flash microplate reader (Thermo Electron Corporation).

H_2O_2 Susceptibility Assay

Overnight cultures of the *P. aeruginosa* strains were diluted to an OD_{600} of 0.05 and cultured at 37°C. When the OD_{600} reached 0.3 (about 1.5–2 h later), AZM (0.5 μg/ml) was added if needed. When the OD_{600} reached 2.0 (about 3 h later), bacteria from 500 μl culture were collected and washed twice with phosphate buffered saline (PBS). Then the bacteria were resuspended in PBS with or without 10% H_2O_2 and incubated for 15 min. The live bacterial numbers were determined by serial dilution and plating.

Motility Assay

The swarming motility was tested on modified M9 medium plates supplemented with 0.2% glucose, 1 mM $MgSO_4$, and 0.05% glutamate as the nitrogen source. 0.5% agar was used for solidification. Two microliters of exponential growth phase *P. aeruginosa* was deposited on the plates, then incubated for 18 h at 37°C (Köhler et al., 2007).

Total RNA Isolation and Quantitative Real-Time PCR

Overnight cultures of *P. aeruginosa* strain PA14 and ΔPA3297 were diluted into fresh LB medium to an OD_{600} of 0.05. The bacteria were grown at 37°C with agitation (200 rpm). When the OD_{600} reached 0.3 (about 1.5–2 h later), AZM was added to reach indicated concentrations. Samples were harvested when the OD_{600} reached 2.0 (about 3 h later; Skindersoe et al., 2008). Total RNA was isolated with an RNeasy Minikit (Tiangen Biotech). The cDNA from each RNA sample was synthesized with reverse transcriptase and random primers (Takara). Real-time PCR was performed with SYBR premix Ex Taq (Roche). The conserved hypothetical protein coding gene PA1769 was used as an internal control (Son et al., 2007). The primers used in quantitative real-time PCR were listed in **Table 2**, with a designation of "RT-qPCR."

Statistical Analysis

When indicated, Student's *t*-test (two-tailed) was used to determine whether the deletion of PA3297 resulted in any significant differences compared to the wild-type cells treated with the same concentrations of AZM.

RESULTS

Deficiency of PA3297 Intensifies the Effects of AZM on Pyocyanin Production

As pyocanin production is suppressed by AZM (Molinari et al., 1992, 1993; Tateda et al., 2001; Favre-Bonté et al., 2003; Gillis and Iglewski, 2004), which can be easily observed and quantified, we used this phenotype to test whether DExD/H box RNA helicases are involved in bacterial response to AZM treatment. There are 17 DExD/H box RNA helicases in the genome of *P. aeruginosa* strain PA14 (www.pseudomonas.com; Winsor et al., 2011). There are nine DExD/H box helicase mutants in the non-redundant PA14 transposon mutants library, however, the other eight DExD/H box helicase mutants are not available (**Table 1**; Liberati et al., 2006; Breidenstein et al., 2011). Thus, we examined pyocyanin production of the available mutants in the absence and presence of AZM at an OD_{600} of 2.0. A PA3297::Tn mutant displayed a significant decrease in pyocyanin production in the presence of AZM, whereas no difference was observed in the absence of AZM (**Figure 1A**). To confirm the role of PA3297, we generated an in frame deletion of PA3297 in PA14. The wild type PA14 and the ΔPA3297 mutant were grown in the absence or presence of 2, 5, or 10 μg/ml AZM. When the bacteria reached same density (OD_{600} of 2.0), we measured the pyocyanin levels. AZM inhibited the production of pyocyanin in the wild type strain in a dose dependent manner. However, the production of pyocyanin by the mutant was repressed more severely in the presence of AZM

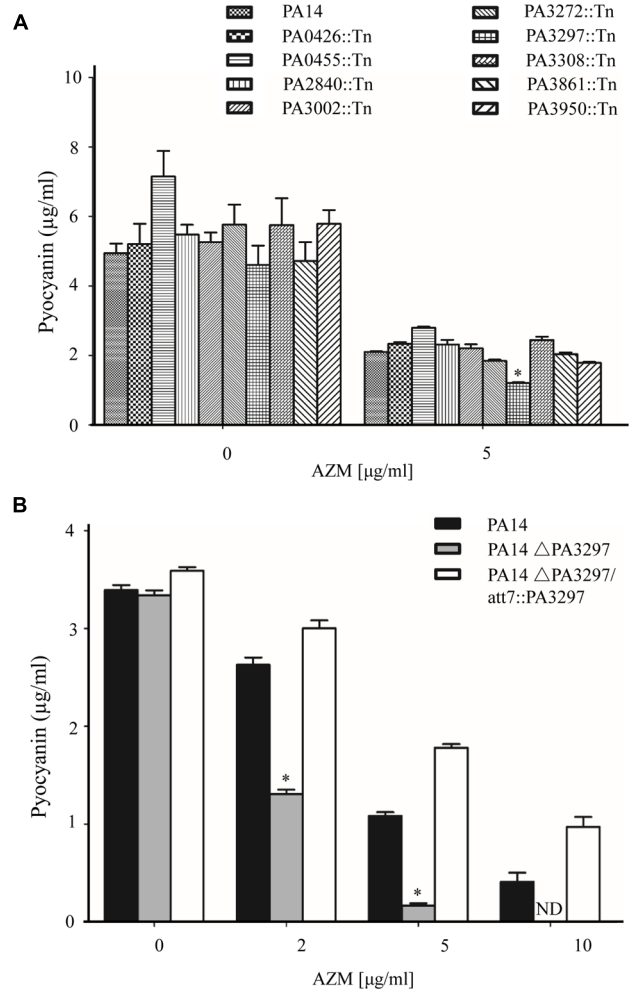

FIGURE 1 | Mutation of PA3297 intensified the AZM mediated inhibition of pyocyanin production. (A) Wild type PA14 and the mutants of DExD/H box helicases were grown to an OD_{600} of 2.0 in the absence or presence of 5 μg/ml AZM at 37°C and the pyocyanin concentrations were measured. **(B)** Bacteria of wild type PA14, ΔPA3297 mutant and the complementation strain were grown in the absence or presence of 2, 5, or 10 μg/ml AZM. When the OD_{600} reached 2.0, the pyocyanin concentrations were measured. The values are the means of three replicates and the error bars display the standard deviations. "ND" standards for "not detected." $^*p < 0.05$ compared to PA14 or the complemented strain by student's *t*-test.

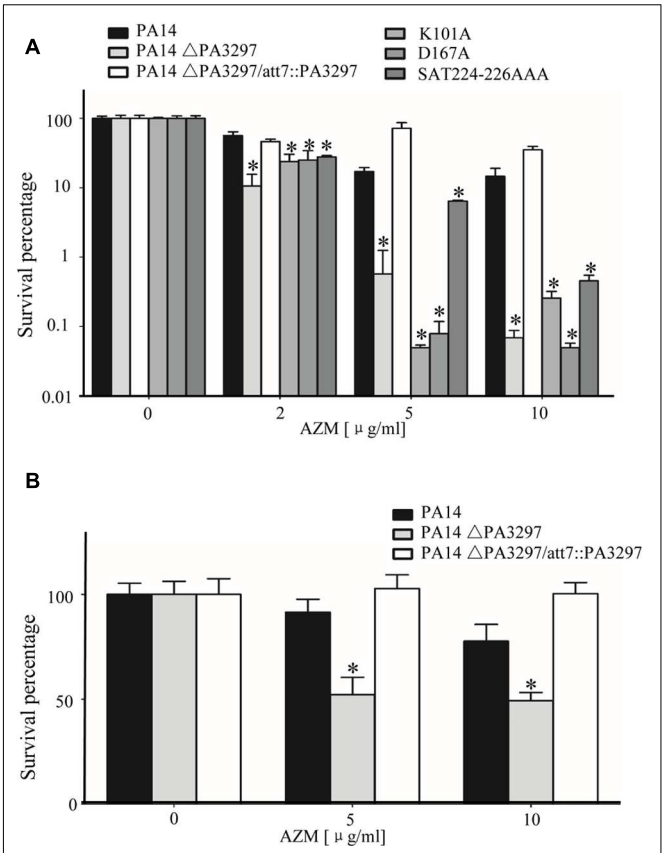

FIGURE 2 | Increased killing of the ΔPA3297 mutant by AZM. (A) AZM-mediated killing of wild type PA14, the ΔPA3297 mutant and the mutant complemented with wild type or the mutated PA3297. Stationary-phase bacteria were treated with 2, 5, or 10 μg/ml AZM for 20 h at 37°C. The numbers of live bacteria were determined by serial dilution and plating. **(B)** AZM-mediated killing of biofilm. 24-h-old biofilms formed by wild type PA14, the ΔPA3297 mutant and the complemented strain were treated with 5 μg/ml, 10 μg/ml AZM or not for 2 h. Bacteria in the biofilm were dissociated from the wells by gentle sonication. The viable bacteria were determined by serial dilution and plating. The averages and associated standard deviations from three replicates are shown. $^*p < 0.05$ compared to PA14 or the complemented strain by student's *t*-test.

at all the tested concentrations (**Figure 1B**). Complementation with a PA3297 gene restored the production of pyocyanin (**Figure 1B**).

Increased Killing of the ΔPA3297 Mutant by AZM

Stationary growth phase *P. aeruginosa* cells are susceptible to AZM (Lovmar et al., 2004, 2009; Imamura et al., 2005; Köhler et al., 2007; Starosta et al., 2010; Gödeke et al., 2013). To test whether mutation of PA3297 renders higher susceptibility, we performed the stationary-phase cells killing assay as previously described (Imamura et al., 2005; Köhler et al., 2007; Gödeke et al., 2013). Stationary-phase cells of PA14, the ΔPA3297 mutant and the complementation strain were subjected to treatment with AZM at the concentrations of 2, 5, and 10 μg/ml. As shown in **Figure 2A**, the AZM-mediated killing of the ΔPA3297 mutant was significantly increased at all of the AZM concentrations tested. The most significant difference was observed at the concentration of 10 μg/ml, where the survival rate of the mutant was approximately 1% of those of the wild type and complemented strains. In addition, the ΔPA3297 mutant was more susceptible to another macrolid antibiotic, erythromycin, with a fourfold lower MIC compared to the wild type strain PA14 (**Table 3**).

Next, we tested the susceptibility of the ΔPA3297 mutant to lincosamides, whose bactericidal mechanism is similar to

macrolides (Tenson et al., 2003; Wilson, 2014). Indeed, mutation in PA3297 increased the bacterial susceptibility to lincomycin and clindamycin (**Table 3**). However, no increase of susceptibility was observed to other antibiotics, including ciprofloxacin, carbenicillin, meropenem, tetracycline, tobramycin, kanamycin, chloramphenicol, or polymyxin B (Supplementary Table S1). These results suggest that PA3297 plays an important role in the resistance against antibiotics targeting the peptide exit tunnel of ribosome.

PA3297 is a putative RNA helicase belonging to the DEAH-box family proteins, which are characterized by the presence of seven to nine conserved motifs (Tanner and Linder, 2001). In the *E. coli* RNA helicases DbpA and HrpA, the conserved residues GETGSGKT in motifI, DEAH in motifII, and SAT in motifIII have been shown to be required for interaction with and hydrolysis of NTP (Koo et al., 2004; Linder and Fuller-Pace, 2013). To determine whether these critical residues within motifs I, II, and III are important for PA3297 in the resistance to macrolides and lincosamides, we altered the residues by site-directed mutagenesis. Specifically, the K101 in motif I or the D167 in motif II was mutated to alanine. The S224 and T226 in motif III were both replaced with alanine. Each mutated PA3297 was transferred into the ΔPA3297 mutant and the susceptibility to antibiotics was tested. None of the mutated PA3297 was able to restore the survival rate of the mutant (**Figure 2A**). In addition, the mutated PA3297 was unable to restore the resistance of the ΔPA3297 mutant to macrolides, lincomycin, and clindamycin (**Table 3**). These results suggest that the RNA helicase function of PA3297 is required for its role in the resistance to macrolides and lincosamides.

Clinically, AZM has been used in the treatment of chronic *P. aeruginosa* infection (Saiman et al., 2003; Blasi et al., 2010), which is characterized by biofilm formation (Singh et al., 2000). The biofilm is notorious for high antibiotic tolerance, which severely hinders eradication of the bacteria (López et al., 2010; Breidenstein et al., 2011). We suspected that mutation of PA3297 might increase the killing efficacy of AZM on the biofilm. Indeed, the survival rates of the ΔPA3297 mutant in biofilm were lower than those of the wild type PA14 at various AZM concentrations, which were restored by complementation with a wild type PA3297 (**Figure 2B**).

TABLE 3 | Bacterial susceptibilities to macrolides and lincosamides.

Strain	MIC (μg/ml)[a]			
	ERY	AZM	LIN	CLI
PA14	300	400	12800	4800
ΔPA3297	75	100	3200	2400
ΔPA3297/att7::PA3297	300	400	>12800	9600
ΔPA3297/att7::PA3297 K101A[b]	75	100	6400	1600
ΔPA3297/att7::PA3297 D192A[b]	75	100	3200	2400
ΔPA3297/att7::PA3297 SAT224AAA[b]	75	100	6400	2400

[a]*ERY, erythromycin; AZM, azithromycin; LIN, lincomycin; CLI, clindamycin.*
[b]*Mutants with single or triple amino acid changes.*

Mutation of PA3297 Increases the Bacterial Susceptibility to Hydrogen Peroxide in the Presence of AZM

It has been shown that AZM treatment impairs the oxidative stress response in *P. aeruginosa* (Nalca et al., 2006), which intrigued us to test whether mutation of PA3297 leads to further impairment. The H_2O_2 susceptibility assay was performed in the presence of 0.5 μg/ml AZM, as the growth rates of wild type PA14 and the ΔPA3297 mutant were similar at this concentration of AZM (Supplementary Figure S1). The wild type PA14, ΔPA3297 mutant and the complemented strain were grown without or with AZM to an OD_{600} of 2.0, and then treated with 10% H_2O_2 for 15 min. In the presence of AZM, the ΔPA3297 mutant was more susceptible to H_2O_2 than the wild type and complemented strains, whereas no difference was observed in the absence of AZM (**Figure 3**). These results suggest that PA3297 is involved in the bacterial oxidative stress response in the presence of AZM.

Mutation of PA3297 Intensifies the Inhibitory Effect of AZM on Swarming Motility

Besides oxidative stress response, AZM suppresses swarming motility (Tateda et al., 2001; Köhler et al., 2007; Gödeke et al., 2013). Same numbers of wild type PA14 and the ΔPA3297 mutant were inoculated on the plates containing various concentrations of AZM. As shown in **Figure 4**, 10 μg/ml AZM suppressed the swarming motility of wild type PA14 obviously, whereas the lower concentrations of AZM showed no inhibitory effect. However, starting from 2 μg/ml, AZM suppressed the swarming motility of the ΔPA3297 mutant in a dose dependent manner. Complementation with a PA3297 gene restored the swarming motility in the presence of AZM (**Figure 4**).

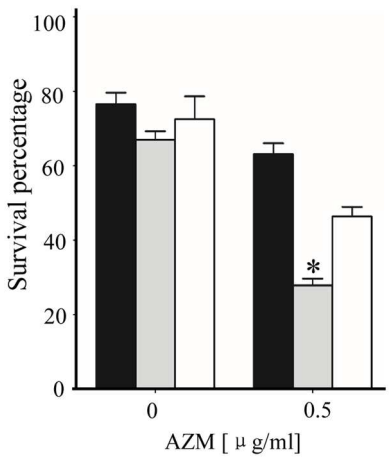

FIGURE 3 | Hydrogen peroxide (H_2O_2) mediated killing of bacteria. PA14, the ΔPA3297 mutant and complemented strain were grown at 37°C in the absence or presence of 0.5 μg/ml AZM to an OD_{600} of 2.0. Bacteria were collected and washed with PBS. Then the bacteria were incubated in PBS with or without 10% H_2O_2 for 15 min. The live bacterial numbers were determined by serial dilution and plating. *$p < 0.05$ compared to PA14 or the complemented strain by student's *t*-test.

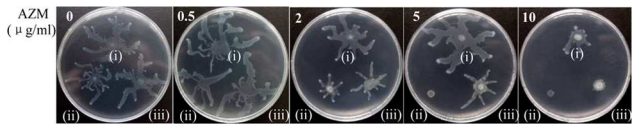

FIGURE 4 | Bacterial swarming motility. PA14 (i), the ΔPA3297 mutant (ii) and the complemented strain (iii) were inoculated on plates containing indicated concentrations of AZM.

Interaction between AZM and Ribosome Induces the Expression of PA3297

Our results so far suggested that PA3297 is involved in the bacterial response to AZM treatment. To test whether the expression of PA3297 is induced by AZM treatment, wild type PA14 was grown in the absence or presence of AZM at various concentrations and the relative RNA levels of PA3297 were determined by real time PCR. Indeed, the expression of PA3297 was induced by AZM (**Figure 5A**). However, overexpression of PA3297 in wild type PA14 did not further increase the bacterial tolerance to AZM (**Table 4**). We suspect that since the bacteria at exponential growth phase are highly resistant to macrolides and lincosamides, overexpression of PA3297 might not further increase the resistance significantly in the MIC test. Another possibility is that with the endogenous up regulation of PA3297, additional expression of PA3297 might be redundant.

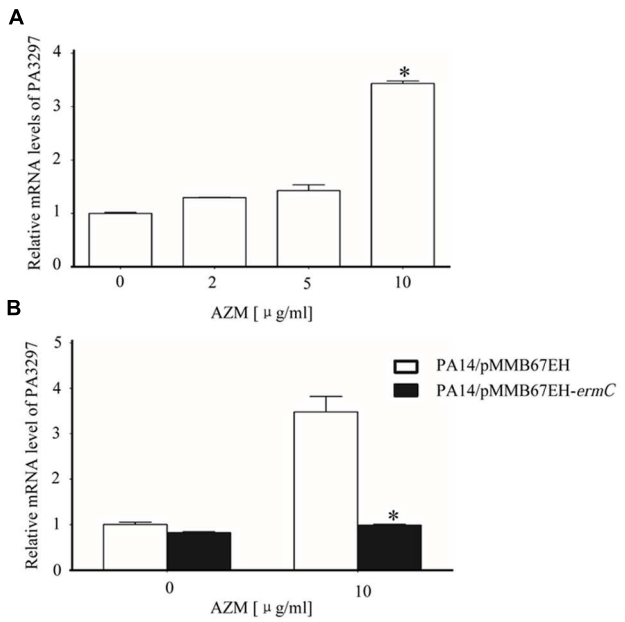

FIGURE 5 | Expression of PA3297 in the presence of AZM. (A) Wild type PA14 was grown to an OD_{600} of 0.3 in LB medium. The bacteria were grown further in the absence or presence of 2, 5, or 10 μg/ml AZM. When the OD_{600} reached 2.0, total bacterial RNA was isolated and the mRNA levels of PA3297 were determined with real time PCR. $^*p < 0.05$ compared to bacteria in the absence of presence of 2 or 5 μg/ml AZM by student's *t*-test. **(B)** PA14 harboring pMMB67EH or the ErmC over expressing plasmid (pMMB67EH-*ermC*) was grown at 37°Cin the absence of AZM. When the OD_{600} reached 0.3, 1 mM IPTG was added to the medium. Meanwhile, no AZM or AZM at the final concentration of 10 μg/ml was added to the medium. At the OD_{600} of 2.0, total RNA was isolated and the mRNA levels of PA3297 were determined with real time PCR. The mRNA levels of *lacI* from the plasmid were used as internal control. $^*p < 0.05$ compared to PA14/pMMB67EH by student's *t*-test.

TABLE 4 | Bacterial susceptibilities to macrolides and lincosamides.

Strain	MIC (μg/ml)[a]			
	ERY	AZM	LIN	CLI
PA14/pMMB67EH	600	200	12800	12800
PA14/pMMB67-PA3297	600	200	12800	6400
PA14/pMMB67-*ermC*	2400	800	>12800	>12800
ΔPA3297/pMMB67EH	75	50	6400	3200
ΔPA3297/pMMB67-PA3297	300	200	12800	3200
ΔPA3297/pMMB67-*ermC*	2400	400	>12800	>12800

[a]*ERY, erythromycin; AZM, azithromycin; LIN, lincomycin; CLI, clindamycin.*

Köhler et al. (2007) previously demonstrated that ribosome is the only target of AZM in bacteria. To test whether the induction of PA3297 is caused by the interaction between AZM and ribosome, we performed the ribosomal protection assay by overexpressing ErmC, a 23S rRNA methylase that blocks the binding of macrolide antibiotics to the NPET (Köhler et al., 2007; Lawrence et al., 2008). In the presence of 10 μg/ml AZM, the growth speed of the ErmC overexpressing strain was similar with that of the wild type strain containing an empty vector. However, overexpression of ErmC abolished the induction of PA3297 by AZM (**Figure 5B**). In addition, antibiotics in the other categories, including ciprofloxacin, tobramycin, and carbenicillin did not affect the expression level of PA3297 (Supplementary Figure S2). These results suggest that the expression of PA3297 is regulated in response to AZM-mediated ribosome stalling.

Ribosome Protection Rescues the ΔPA3297 Mutant from AZM-Mediated Hyperlethality

So far, we have demonstrated that the expression of PA3297 is induced by AZM and that mutation of PA3297 renders *P. aeruginosa* hypersusceptible to AZM. These results suggest that PA3297 might play a role in counteracting the detrimental effects caused by the interaction between AZM and ribosome (Köhler et al., 2007; Gödeke et al., 2013). Thus, ribosome protection should be able to increase the tolerance of the ΔPA3297 mutant to AZM. Indeed, overexpression of ErmC increased both the growth speed and the survival rate of the ΔPA3297 mutant when treated with 10 or 40 μg/ml AZM (Supplementary Figure S3, **Figure 6**). In addition, overexpression of ErmC increased the MICs of both wild type PA14 and the ΔPA3297 mutant to AZM, erythromycin, lincomycin and clindamycin (**Table 4**). Mutation of PA3297 did not increase the bacterial susceptibility to a variety of other antibiotics (Supplementary Table S1). These results suggest a specific role of PA3297 in responding to lincosamides and macrolides.

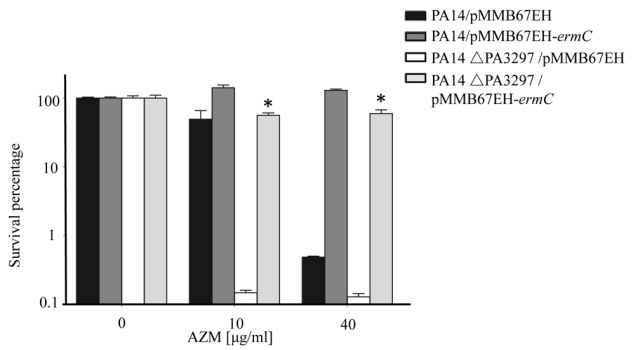

FIGURE 6 | Effects of Ribosome protection on the AZM mediated killing of the ΔPA3297 mutant. Wild type PA14 or the ΔPA3297 mutant harboring pMMB67EH or pMMB67EH-*ermC* was grown to an OD_{600} of 0.5 when 1 mM IPTG was added to the medium. When the OD_{600} reached 2.0, the bacteria were treated without or with 10 or 40 μg/ml AZM for 20 h at 37°C. The numbers of live bacteria were determined by serial dilution and plating. *$p < 0.05$ compared to the ΔPA3297 mutant harboring pMMB67EH by student's *t*-test.

Deficiency in PA3297 Compromises rRNA Processing in the Presence of AZM

Studies in *E. coli* demonstrated that the DExD/H box play crucial roles in rRNA processing (Iost et al., 2013; Linder and Fuller-Pace, 2013). Therefore, we suspected that the up regulated PA3297 might participate in rRNA maturation, which facilitates ribosome biogenesis to compensate for AZM inactivated ribosome. The rRNA coding region in the chromosome of *P. aeruginosa* PA14 is shown in Supplementary Figure S4. To examine the processing of the rRNA transcript, we designed real-time PCR primers to analyze the total 23S and 5S rRNA levels as well as primers across the 23S and 5S rRNA coding region to analyze the level of unprocessed rRNA (Supplementary Figure S4). In wild type PA14, AZM at the concentrations of 5 and 10 μg/ml reduced the 23S and 5S rRNA levels (**Figures 7A,B**), and 10 μg/ml AZM slightly increased the unprocessed 23S-5S rRNA level (**Figure 7C**). In the absence of AZM, the total 23S and 5S rRNA levels were higher in the ΔPA3297 mutant than those in the wild type strain (**Figures 7A,B**), whereas the unprocessed 23S-5S levels were similar between the mutant and wild type strain. Interestingly, treatment with 10 μg/ml AZM resulted in a higher level of unprocessed 23S-5S rRNA in the ΔPA3297 mutant (**Figure 7C**), although its total 23S and 5S rRNA levels were lower than those in the wild type strain (**Figures 7A,B**).

Next, we calculated the percentages of unprocessed 23S-5S rRNA in wild type PA14 and the ΔPA3297 mutant with or without AZM treatment. Standard curves were generated to determine the amplification efficiencies of the primer pairs for the detection of total 23S, 5S and unprocessed 23S-5S rRNA levels in real time PCR (Supplementary Figure S5). Considering 5S rRNA is more prone to be lost during RNA purification, we calculated the ratio of unprocessed 23S-5S rRNA by dividing the levels of 23S-5S rRNA by those of 23S rRNA calibrated with the amplification efficiencies (**Table 5**). In the absence of AZM, the

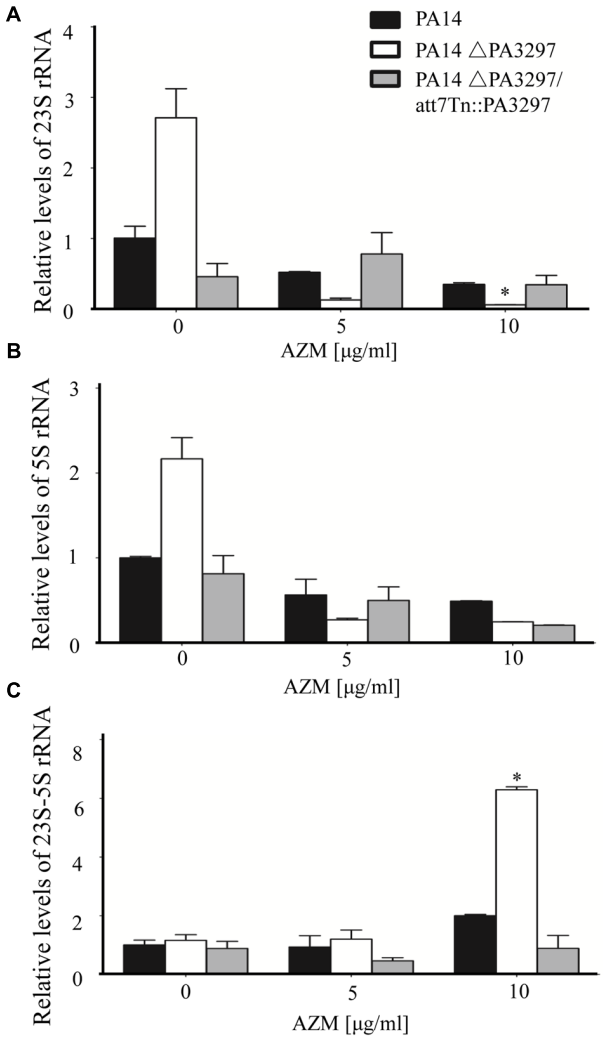

FIGURE 7 | Effects of AZM on the processing of rRNA. PA14, the ΔPA3297 mutant and the complemented strain were grown at 37°C in the absence of AZM. When the OD_{600} reached 0.3, AZM (0, 5, and 10 μg/ml) was added. Total RNA was harvested when the OD_{600} reached 2.0. The levels of total 23S **(A)**, 5S **(B)**, and unprocessed 23S-5S rRNA **(C)** were determined by real time PCR. The mRNA levels of PA1769 were used as an internal control. *$p < 0.05$ compared to PA14 by student's *t*-test.

ratios of unprocessed 23S-5S rRNA were approximately 0.014 and 0.009% in wild type PA14 and the ΔPA3297 mutant, respectively. It seems that, without PA3297, the processing of 23S-5S rRNA is even more efficient. In the presence of 5 and 10 μg/ml AZM, the ratios of unprocessed 23S-5S rRNA rose to approximately 0.05 and 0.09% in wild type PA14, respectively. However, the ratios of unprocessed 23S-5S rRNA in the ΔPA3297 mutant were 0.33 and 1.61%, which were approximately 5- and 18-fold higher than those in PA14 under the same condition (**Table 5**).

Since, the growth speed of the ΔPA3297 mutant in the presence of 10 μg/ml AZM was similar as that of the wild type PA14 in the presence of 40 μg/ml AZM (Supplementary Figure S3), we compared the rRNAs levels of the two strains

TABLE 5 | The ratio of unprocessed 23S-5S rRNA in total 23S rRNA (%).

Strain	AZM (μg/ml)		
	0	5	10
PA14	0.014	0.051	0.089
ΔPA3297	0.009	0.335	1.608

The unprocessed 23S-5S rRNA ratios were calculated according to the equation: Ratio = $(1+e_{23S})^{C_t(23S)}/(1+e_{23S\text{-}5S})^{C_t(23S\text{-}5S)}$; e: Amplification efficiency. C_t: Cycles when fluorescence intensity reaches detection threshold.

grown under the two conditions. The relative levels of 23S-5S rRNA, total 5S and 23S rRNA in the ΔPA3297 mutant were 150, 55, and 100% of those in the wild type PA14, respectively, indicating a similar rRNA processing status with the two different AZM concentrations. In combination, these results suggest that PA3297 might contribute to rRNA processing in response to AZM. And mutation of PA3297 might impair the biosynthesis of ribosome under AZM treatment, which renders the bacterium more susceptible to AZM.

DISCUSSION

By binding to 23S rRNA in the 50S subunit of bacterial ribosome, AZM blocks polypeptide elongation and diminishes the intracellular pools of aminoacyl-tRNAs (Tenson et al., 2003; Gödeke et al., 2013; Wilson, 2014). And it has been demonstrated that AZM reduces the expression of *gacA* and the small RNAs *rsmY* and *rsmZ,* as well as quorum sensing genes (Kai et al., 2009; Pérez-Martínez and Haas, 2011). And the stationary phase killing by sub-MIC AZM was demonstrated to be correlated with increased outer membrane permeability (Imamura et al., 2005). Here, we found that mutation of PA3297 intensified the AZM mediated inhibitory effects on pyocyanin production and swarming motility of *P. aeruginosa*. In addition, the PA3297 mutant is more susceptible to oxidative stress in the presence of AZM. During infection, host generated reactive oxygen species (ROS) is an important bacterial killing mechanism. Therefore, inhibition of PA3297 together with the treatment with AZM, might render the bacteria more susceptible to host killing.

The MIC of the ΔPA3297 mutant is a quarter of that of the wild type strain. Consistently, the growth speed of the ΔPA3297 mutant in the presence of 10 μg/ml AZM was similar as that of the wild type strain in the presence of 40 μg/ml AZM (Supplementary Figure S3). However, in the stationary phase cell killing assay, the survival rate of the ΔPA3297 mutant treated with 10 μg/ml AZM was approximately 20% of that of the wild type strain treated with 40 μg/ml AZM (**Figure 6**). Therefore, PA3297 might play a more important role in the survival of stationary phase cells under AZM treatment.

Macrolides and lincosamides bind to the 50S of ribosome and block the NPET (Tenson et al., 2003). In our experiments, mutation in PA3297 did not alter the bacterial resistance to other antibiotics, including those binding to 30S or other parts of 50S ribosome. In addition, the expression of PA3297 was upregulated by AZM, which was abolished by ribosome protection. Therefore, PA3297 might specifically play a role in bacterial response to macrolides and lincosamides.

PA3297 is also named HrpA, both in PAO1 and PA14 (Winsor et al., 2016). According to the Profiles from GEO Expression Database at NCBI, the expression level of PA3297 was higher in biofilm than that in planktonic cells (Anderson et al., 2008). Isolates from CF lungs displayed higher expression levels of PA3297 levels than PAO1 (Son et al., 2007; Bielecki et al., 2013). And artificial medium that mimics CF lung sputum could increase the expression level of PA3297 slightly (Fung et al., 2010). In addition, increase of PA3297 expression was also observed in antibiotic-resistant small colony variants (Wei et al., 2011). However, the expression level of PA3297 showed a significant decrease in response to airway epithelia or low oxygen conditions (Alvarez-Ortega and Harwood, 2007; Chugani and Greenberg, 2007). These results indicate that the expression of PA3297 is regulated in response to various environmental stesses.

PA3297 is predicted to locate at the cytoplasmic membrane, with a molecular weight of 149.8 kDa (Winsor et al., 2016). It belongs to the DExD/H box helicase family. Members of this family have been found to play crucial roles in RNA metabolism and gene regulation (Linder and Jankowsky, 2011; Iost et al., 2013; Kaberdin and Bläsi, 2013; Linder and Fuller-Pace, 2013; Putnam and Jankowsky, 2013). According to NCBI protein blast, there are 81 homologous proteins with identities of 78% or more in other microorganisms. The homolog of PA3297 in *Borrelia burgdorferi* was found to be required for mouse infectivity and tick transmission and involved global gene regulation (Salman-Dilgimen et al., 2011; Owttrim, 2013). Another homolog in *E. coli* was found to be involved in fimbrial biogenesis (Koo et al., 2004). The identities they shared with PA3297 are 34 and 49%, respectively. Both of them possess the conserved residues in motif I, motif II, and motif III. Meanwhile, they are also involved in RNA processing. In *Listeria monocytogenes*, it has been reported that defect of a DExD-box RNA helicase, Lmo1722, reduced the maturation of 23S RNA (Bareclev et al., 2014) at low temperatures.

In *E. coli*, the primary transcript rRNA is cleaved by RNase III, yielding precursors of the 16S rRNA (17S rRNA), 23S rRNA, and 5S rRNA (9S rRNA; Shajani et al., 2011). Mutation of RNase III led to slower growth rate, reduction in cell viability and protein synthesis rates in the presence of AZM (Silvers and Champney, 2005). In *E. coli*, under certain stress conditions, DEAD box helicase may substitute for RhlB in the degradosome, such as CsdA under cold shock conditions (Prud'homme-Généreux et al., 2004). We found that mutation of PA3297 increased the percentage of unprocessed 23S-5S rRNA in the presence of AZM, which indicates that the cleaving function of RNase III might be impaired under the stress caused by AZM. Therefore, PA3297 might assist RNase III in rRNA processing in the presence of AZM. In addition, although the growth speed and rRNA processing in the ΔPA3297 mutant treated with 10 μg/ml AZM were similar to those in the wild type strain treated with 40 μg/ml AZM (Supplementary Figure S3), the survival rate of the ΔPA3297 mutant was lower than that of the wild type strain

under those conditions. Thus, PA3297 might play other roles in counteracting the effects of AZM.

Overall, mutation of PA3297 renders *P. aeruginosa* more susceptible to AZM mediated inhibition on virulence factors and killing effect. Therefore, targeting the regulatory pathway or the function of PA3297 might further increase the beneficial effects of AZM in the treatment of chronic *P. aeruginosa* infections.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: WW, SJ, HT, ZC. Performed the experiments: HT, LZ, YW, RC, FZ, YJ. Analyzed the data: HT, LZ, SJ, WW. Wrote the paper: HT, SJ, WW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00317

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Efflux Pump Blockers in Gram-Negative Bacteria: The New Generation of Hydantoin Based-Modulators to Improve Antibiotic Activity

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Multidrug resistant (MDR) bacteria are an increasing health problem with the shortage of new active antibiotic agents. Among effective mechanisms that contribute to the spread of MDR Gram-negative bacteria are drug efflux pumps that expel clinically important antibiotic classes out of the cell. Drug pumps are attractive targets to restore the susceptibility toward the expelled antibiotics by impairing their efflux activity. Arylhydantoin derivatives were investigated for their potentiation of activities of selected antibiotics described as efflux substrates in *Enterobacter aerogenes* expressing or not AcrAB pump. Several compounds increased the bacterial susceptibility toward nalidixic acid, chloramphenicol and sparfloxacin and were further pharmacomodulated to obtain a better activity against the AcrAB producing bacteria.

Keywords: antibiotics, AcrAB pump, efflux pump blocker, hydantoin, multidrug resistance

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INTRODUCTION

Multidrug resistance (MDR) described in Gram-negative bacteria is continuously emerging as a prominent worldwide health concern (Chopra et al., 2008; Laxminarayan et al., 2008; Gandhi et al., 2010). One of the most contributing mechanisms is the overexpression of efflux pumps that are involved in bacterial survival, colonization and virulence (Delmar et al., 2014; Blair et al., 2015; Li et al., 2015; Venter et al., 2015; Davin-Regli et al., 2016). Several efflux pump superfamilies, e.g., major-facilitator (MF), multi-drug and toxic efflux (MATE), ATP-binding cassette (ABC), small multidrug resistance (SMR), resistance-nodulation-division (RND) transporters have been classified[1,2] and extensively described in well-documented reviews: they differ by their functional structure and organization, their subcellular location inside the bacterium, their energy source (e.g., membrane potential for RND or ATP for ABC) and the involvement of a coupled antiport during the antibiotic expulsion (e.g., proton for AcrAB pump; Delmar et al., 2014; Blair et al., 2015; Li et al., 2015). The overexpression of Gram-negative efflux pumps, especially those belonging to the

[1] http://www.membranetransport.org/
[2] http://www.tcdb.org/

RND family, is now well-described in resistant isolates (Nikaido and Pagès, 2012; Li et al., 2015). This contributes to the acquisition of additional mechanisms of resistance including the mutation in antibiotic targets (e.g., mutation in gyrase/topoisomerase for quinolone) or the production of enzymes that degrade antibiotics (e.g., β-lactamases) and this can be associated or not with the alteration of the outer membrane permeability (Davin-Regli et al., 2008; Pagès et al., 2008). Regarding resistant clinical isolates of Gram-negative bacteria, the archetype of the drug active transporter system is the AcrAB-TolC/MexAB-OprM efflux pumps (Nikaido and Pagès, 2012; Li et al., 2015). The structures of components of efflux systems belonging to RND group, have been solved by X-ray crystallography and models of the pump assembly have been obtained (Yao et al., 2010; Du et al., 2014). The structure and function of the RND efflux pumps must be molecularly deciphered thus allowing the rational design and the synthesis of new compounds to combat MDR. The broad selectivity of efflux pumps makes difficult the identification of precise pharmacophoric groups at the drug surface. However, efflux pumps are attractive target by blocking this efflux mechanism in order to restore the intracellular concentration of antibacterial agents (Bolla et al., 2011; Ruggerone et al., 2013; Dreier and Ruggerone, 2015; Opperman and Nguyen, 2015). Recent computer docking analyses have produced some information about the involvement of certain amino acid residues, but clearly more chemical and biological information are needed to improve models (Schulz et al., 2010; Fischer et al., 2014). This is a 'key' point not only regarding the mode of action and dynamics of the process but also regarding the clinical impact of the design of new antibacterial agents: this last aspect is illustrated by the β-lactamases inhibitors currently used today (Bolla et al., 2011; Chen et al., 2013; Pucci and Bush, 2013).

To enhance the activity of old antibiotics by targeting resistance mechanisms in clinical resistant isolates, they can be combined with adjuvant molecules such as chemosensitizers (e.g., membrane permeabilizer or efflux inhibitor; Jones, 2010; Bolla et al., 2011). In addition, these types of transporter inhibitors may impair the activity of efflux pumps and thus reduce bacterial colonization and virulence (Venter et al., 2015; Davin-Regli et al., 2016). It must be noted that the efficacy of the inhibitors depends on their affinity for transporter binding sites (compared to the antibiotic) and their internal concentration close to the efflux pump. Consequently, due to these parameters associated with penetration rate and affinity for pump sites, some discrepancies can be observed in the level of the internally accumulated antibiotics depending on the bacterial backgrounds (Kaščáková et al., 2012; Cinquin et al., 2015).

Recently, two generations of hydantoin derivatives have been identified as AcrAB-TolC inhibitors with the *Enterobacter aerogenes* CM 64 strain that overproduces AcrAB (Handzlik et al., 2011). Compounds showing chemosensitizing effect on nalidixic acid activity were the starting point for new pharmacomodulations carried out in this study to obtain a new generation of products with an improved activity. Moreover, tests were extended to other chemically unrelated antibiotics, chloramphenicol and sparfloxacin, for which the antibacterial activity decreased together with the emergence of multidrug-resistant strains. Several chemical derivatives were synthesized to define pharmacophoric groups important for restoring the activity of antibiotics in AcrAB active bacteria.

MATERIALS AND METHODS

Bacterial Strains

All generations of hydantoin derivatives were tested against two strains of *E. aerogenes*: the reference strain, ATCC 13048 (basal efflux producer) and its derivative strain, CM 64, a chloramphenicol-selected resistant strain that over-produces the AcrAB-TolC EPs (Ghisalberti et al., 2005). In addition, two isogenic strains EA289 (a clinical MDR strain that overproduced the AcrAB pump) and its EA294 derivative (an acr*AB* knockout strain) were used (Pradel and Pagès, 2002). These two strains contain additional mechanisms of antibiotic resistance such as ß-lactamases, targets mutations, etc. (Malléa et al., 1998; Pradel and Pagès, 2002; Kaščáková et al., 2012).

Chemicals

Hydantoin derivatives used in the pharmacological assays were obtained by chemical synthesis. The new generations IIIA and B were obtained using 3–4-step synthesis (Handzlik et al., 2014; Matys et al., 2015; **Figure 1** and Supplementary data). Purity and identity of new compounds were confirmed using spectral analysis (H-NMR, IR), elemental analysis and melting point measurements. Phenylalanine-Arginine β-naphthylamide (PAβN, dihydrochloride, Sigma) previously described as efflux pump inhibitor was used as reference (Bolla et al., 2011; Misra et al., 2015).

Compound Susceptibility Assays

Susceptibilities of ATCC 13048, CM 64, EA294, and EA289 were determined by using the twofold standard microbroth dilution method (microplates and automatic analyses Tecan®; CLSI[3]). Approximately, 10^5 CFU (colony forming unit) were inoculated in 200 μl of Mueller-Hinton II broth (MH II broth cation adjusted, Becton, Dickinson & Company) containing twofold serial dilutions of the targeted molecule. Experiments were performed in triplicate for each compound and each antibiotic. Results were estimated visually after 18 h incubation at 37°C (Philippe et al., 2015).

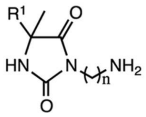
Antibiotic Susceptibility Potentiating Assays

To assay the possible chemosensitizing activity of compounds, serial dilutions of antibiotics, nalidixic acid (NAL, Sigma), chloramphenicol (CHL, Sigma), doxycycline (DOX, hyclate, Sigma), erythromycin (ERY, lactobionate, AMDIPHARM) and

[3]http://clsi.org/

Generation IIIA

Compound	R^1	n
29		3
30		4
31		5
32		4



Generation IIIB

Compound	R^1
33	H
34	$-CH_3$
35	$-CH_2COOC_2H_5$
36	

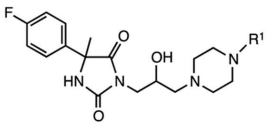


FIGURE 1 | Compounds of generation IIIA and generation IIIB of hydantoins.

sparfloxacin (SPX, Sigma), were incubated in the absence or in the presence of compounds. The antibiotics, NAL, CHL, and SPX are substrates of the AcrAB-TolC efflux pump as demonstrated by the increased MIC values obtained in CM 64 strain overexpressing the AcrAB-TolC pump (**Table 1**) compared to the reference one ATCC 13048. Thus we are able to hypothesize that an efflux blocker may reduce the antibiotic MIC in efflux producer strain. Generations IIIA and IIIB of hydantoin derivatives were tested at a concentration of 0.5 mM according to the intrinsic antibacterial activity of each compound (corresponding to the value of MIC/4). To facilitate the comparison of activity and the performance of a rational SAR analysis, they were additionally tested at the concentration corresponding to that of the best first generation of chemosensitizers (0.0625 mM; Handzlik et al., 2011). PAβN, the reference inhibitor for AcrAB pump, was used at 0.050 mM. Control experiments were carried out without compounds. Experiments were performed in triplicate for each antibiotic, each strain and each condition (without and with compound). The results were assessed after 18 h at 37°C and were presented by using the activity gain parameter A, the ratio of the MIC of the antibiotic (determined in the absence of compound) to its MIC in the presence of the compound.

TABLE 1 | Susceptibility (MIC) of the *Enterobacter aerogenes* reference strain ATCC 13048, the derivative strain CM 64 overexpressing AcrAB-TolC pump, Ea289 overproducing the AcrAB pump and its derivative Ea294 (an acr*AB* knockout strain) to the different compounds belonging to various generations of hydantoins and to nalidixic acid (NAL), chloramphenicol (CHL), sparfloxacin (SPX), doxycycline (DOX), and erythromycin (ERY).

Compound	MIC [mM] ATCC 13048	MIC [mM] CM 64	MIC [mM] Ea294	MIC [mM] Ea289
29–36	>2	>2	>1	>1
NAL	0.034	0.55 (16)*	4.4	>17.6
CHL	0.012	0.79 (66)	0.2	3.2
SPX	0.00015	0.0025 (17)	>2	>2
DOX	0.002	0.07 (35)	0.002	0.07
ERY	0.35	0.7 (2)	0.087	0.35
PAβN	5	5 (1)	0.0625	3.5

**MIC ratio: efflux overproducer/basal producer strains.*

Determination of the FIC Index

To determine the fractional inhibitory concentration index (FICi), a two dimensional checkerboard with twofold dilutions of each compound was set up for the study (Berenbaum, 1978;

TABLE 2 | Effect of the hydantoin derivatives on the susceptibility level of *E. aerogenes* ATCC 13048 and CM 64 strains to nalidixic acid (NAL), chloramphenicol (CHL), and sparfloxacin (SPX).

Compound	Concentration [mM]	A_{NAL}		A_{CHL}		A_{SPX}	
		ATCC 13048	CM 64	ATCC 13048	CM 64	ATCC 13048	CM 64
29	0.0625	1	1	1	1	2	2
30	"	2	2	2	1	1	1
31	"	2	2	2	1	1	2
32	"	2	4	1	1	1	2
33	"	1	1	1	1	1	1
34	"	1	0.5	0.5	1	0.5	1
35	"	1	1	0.5	1	0.5	0.5
36	"	1	1	1	1	0.5	0.5
29	0.5	8	4	2	4	4	2
30	"	8	4	4	4	8	4
31	"	16	4	4	4	4	4
32	"	16	32	4	32	8	8
33	"	2	2	1	2	2	1
34	"	2	1	1	1	2	2
35	"	2	1	2	1	2	1
36	"	2	1	2	1	2	1
PAβN	0.05	64	128	2	64	8	32

"A" corresponds to the antibacterial activity gain obtained in the presence of the respective compound and evaluated for each antibiotic (A = MIC without compound/MIC with compound).

Allam et al., 2014). For the first clear well in each row of the microplate containing an antimicrobial agent, the FIC was calculated as follows: FIC of compound A (FIC A) = MIC of compound A in combination with B/MIC of compound A alone, and FIC of compound B (FIC B) = MIC of compound B in combination with A/MIC of compound B alone. The FICi was calculated as the sum of the FIC of each compound. The nature of the interaction was classified as follows: synergy FICi $\leq$ 0.5; additivity 0.5 $<$ FICi $\leq$ 1; indifference 1 $<$ FICi $\leq$ 2; and antagonism FICi $>$ 2 (European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), 2000). For each combination, an isobologram, which graphically illustrates the interaction effect, was constructed: FIC A was showed in the abscissa and FIC B in the ordinate, the profile of the corresponding curve reflects the nature of the interaction.

RESULTS

Antibacterial Activity

It is important that putative inhibitors that could be used as "adjuvant molecule" for antibiotics, do not display a high intrinsic antibacterial activity (Davin-Regli et al., 2008; Bolla et al., 2011). The determination of the antibacterial activity for each compound was performed and presented in **Table 1**. Many of the compounds did not inhibit the growth of bacteria even at the highest tested concentration as the previous generations of hydantoin derivatives (Handzlik et al., 2011). The majority of compounds exhibited a MIC $>$ 2 mM in ATCC13048 and in CM64 strains and MIC $\geq$ 1 mM in Ea289 and Ea294.

Influence on Antibiotic Susceptibility in ATCC 13048 and CM 64 Strains

Effect on Nalidixic Acid Susceptibility

Table 2 presents the chemosensitizing effect of compounds on ATCC 13048 and CM 64 susceptibility to nalidixic acid (NAL). The gain on antibiotic activity (A) was calculated for each compound and each antibiotic. Regarding the CM 64 strain, a moderate or a weak effect was observed on the MIC when compounds were used at 0.0625 mM compared to PAβN. Compounds of generations IIIA (**29**–**32**) and IIIB (**33**–**36**) were used at a concentration of 0.0625 mM and 0.5 mM due to their higher MIC. A higher concentration of compounds of the generation IIIB did not improve the antibiotic activity whereas we observed a significant decrease of the antibiotic MIC, from 4- to 32-fold in CM 64 (see A_{NAL} in **Table 2**), in the case of compounds of generation IIIA (**29**–**32**). These compounds decreased the MIC of NAL from 8- to 16-fold comparing to the PAβN effect (64-fold) in the reference ATCC 13048 strain (see A_{NAL} in **Table 2**). In the case of PAβN and derivatives **32** the difference in activity in both tested strains was not significant (only a twofold stronger activity in the strain overexpressing the AcrAB pump than in the reference one) whereas compounds **29**–**31** showed a better action on antibiotic activity in the reference strain. It must be noted that the compound **32** used at increased concentration exhibits an activity profile similar to PAßN, which has been shown to be an efficient efflux inhibitor at low concentrations (Nikaido and Pagès, 2012; Misra et al., 2015).

TABLE 3 | Effect of the compounds of generation IIIA (29–32) depends on AcrAB context.

Compound	Ea294			Ea289			CM 64	
	A_{DOX}	A_{ERY}	A_{NAL}	A_{DOX}	A_{ERY}	A_{NAL}	A_{DOX}	A_{ERY}
29	2	1	2	8	1	1	8	1
30	4	4	4	16	1	2	8	1
31	4	8	8	16	2	2	8	1
32	2	2	4	32	4	>2	32	2

"A" corresponds to the antibacterial activity gain obtained in the presence of the respective compound and evaluated for each antibiotic (A = MIC without compound/MIC with compound). DOX, doxycycline; ERY, erythromycin; NAL, nalidixic acid.

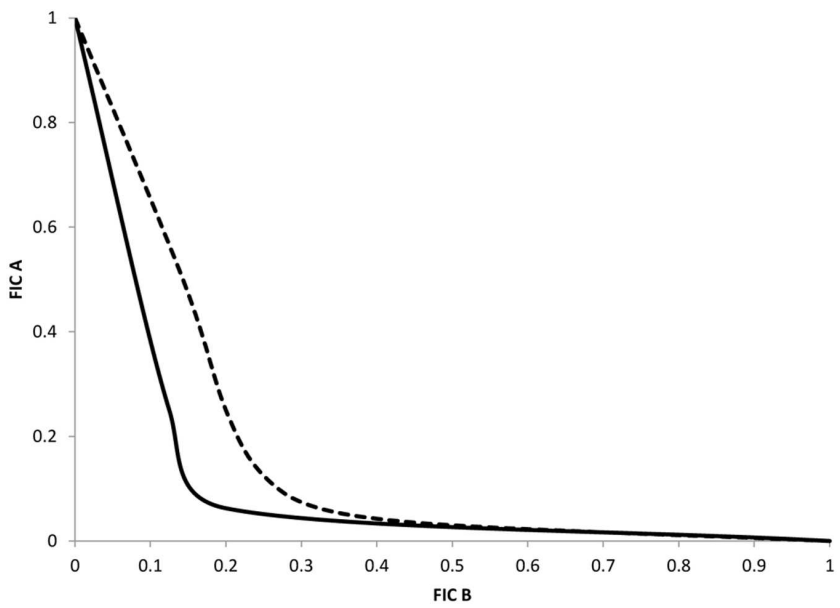


FIGURE 2 | Representative isobologram of interactions between compound 32 and nalidixic acid or chloramphenicol. Nalidixic acid, solid line; chloramphenicol, dotted line. The axis numbers correspond to normalized FICs obtained with *Enterobacter aerogenes* CM64 strain.

Effect on Chloramphenicol Susceptibility

The results are presented in **Table 2**. In the case of the CM 64 strain, compounds tested at the concentration of 0.0625 mM showed a weak chemosensitizing effect on CHL antibacterial activity compared to PAβN. Compounds of generation IIIA (**29**–**32**) examined at a concentration of 0.5 mM decreased the chloramphenicol MIC from 2- to 4-fold in the reference strain and from 4- to 32-fold in the strain overexpressing efflux pump (see A_{CHL} in **Table 2**). We observed a noticeable increase in CHL susceptibility in the AcrAB overproducer CM 64, a 32-fold gain in the susceptibility with the compound **32** compared to compounds **29–31.** In this assay, the chemosensitizing effect of compound **32** can be compared to PAβN which was much more active in the strain overexpressing the AcrAB pump than in the parental strain ATCC 13048.

Effect on Sparfloxacin Susceptibility

Table 2 presents the effects of hydantoins on sparfloxacin (SPX) susceptibility. In CM 64, the chemosensitizing effect of low concentrated hydantoins on SPX was as weak as we observed in the case of CHL. Compounds of generation IIIA (**29**–**32**) tested at the highest concentration 0.5 mM caused a 4–8-fold decrease in the MIC in the ATCC 13048 strain and a 2–8-fold decrease in the MIC in CM 64 one. Comparing the results obtained for the active compounds (**29–32**) to the results obtained for PAβN (see A_{SPX} in **Table 2**) we observed that the action of hydantoins with SPX was less efficient than PAβN, which was more active in the CM64 strain overexpressing the AcrAB pump than in the reference strain ATCC13048. This may suggest a different conformational site for the two molecules, either for recognition or for binding step, in the AcrB monomer (Delmar et al., 2014; Du et al., 2014; Yamaguchi et al., 2015), inside the pump or a different mode of action on the resistance mechanism.

Effect of Compounds 29–32 on the Resistance Level in MDR *E. aerogenes* Strains

In order to evaluate the chemosensitizing effect of the compounds **29**–**32** (the most effective molecules) on the MDR

background, the Ea289 and Ea294 strains were assayed. It is important to mention that these strains contain various resistance mechanisms (Malléa et al., 1998; Pradel and Pagès, 2002) such as target mutations (e.g., mutations in QRDR region of gyrase that increase quinolone resistance) and expression of modifying enzymes (e.g., ß-lactamases that contribute to ß-lactam resistance). **Table 3** presents the activity gain parameter A tested with doxycycline (DOX), erythromycin (ERY), and nalidixic acid (NAL) in parental and AcrAB-derivative strain context. It is interesting to note that **32** exhibited significant restoring antibiotic activity with DOX in Ea289 and very weak action in Ea294 that is devoid of the AcrAB efflux pump components. In contrast, a weak chemosensitizing activity was observed with ERY, this effect could be caused by the presence of additional resistance mechanisms for the macrolide antibiotic class as previously reported (Chollet et al., 2004). Regarding the effect on NAL and sparfloxacin susceptibility, the mutations in the quinolone target (DNA gyrase) previously reported in Ea289, can explain the weak effect observed toward these strains (Pradel and Pagès, 2002; Kaščáková et al., 2012).

Determination of the FIC Index for Compound 32

To precise the type of interaction (synergistic, additive, or indifferent) between compound **32** and selected antibiotics, we carried out analysis based on the FICi as previously described. Combinations of compound **32** with NAL and CHL respectively were performed in CM 64 strain. This strain overexpresses the AcrAB pump and does not contain target mutation that can impair the effect of compounds on the restoration of antibiotic activity. The nature of the association was determined from the FICi average obtained from each combination and the representation was performed for each combination (**Figure 2**). In two cases, the isobologram curve obtained was concave indicating a synergy association between antibiotic and compound **32**. The synergistic association corresponds to an average of calculated FICi of about 0.32 for NAL and 0.44 for CHL respectively. These curves fitted well with the **Table 2** data.

DISCUSSION

The aim of this study was to identify compounds that are able to modulate the AcrAB pump activity and restore antibiotic activity on efflux producing strains.

The various molecules combined with NAL and CHL increased the susceptibility of the CM 64 strain and of the reference strain ATCC 13048 to antibiotics. On the one hand, we found compounds that showed stronger activity in the ATCC 13048 strain than in the CM 64 strain (**29–31** tested with NAL, **29, 30** tested with SPX). These findings could indicate in this case that tested compounds are not highly selective toward the AcrAB-TolC pump involved in the efflux of antibiotics. This suggests that they act not only on the basal AcrAB expressing strain but also that they may recognize other bacterial targets. In contrast, PAβN combined with CHL and SPX as well as the 3-aminobutyl-5-β-naphthylhydantoin **32** tested with CHL exhibited a difference between the AcrAB overproducer strain and the parental one. This time these results suggest a significant selectivity of chemosensitizers for the AcrAB pump expressed in bacterial strains. Taking into account the influence of concentration of 3-aminoalkyl-5-naphthylhydantoins (generation IIIA) on the antibiotic activity, we observed that the higher is the concentration, the stronger is the chemosensitizing effect, not only on the CM 64 strain but also on ATCC 13048. These outcomes could suggest additional mechanisms of action besides the effect on the AcrAB-TolC efflux pump.

The comparison of the results in Ea289 and Ea294 (**Table 3**) indicates the correlation of the restoring activity of antibiotics with the presence of the AcrAB efflux pump. They also illustrate the capability of compound **32** to increase the antibacterial effect of DOX in these strains. The low effect of **32** on the ERY susceptibility when assayed in Ea289 and Ea294 can be due to additional pump, other than AcrB, active in these clinical derivative strains capable to expel this class of drugs (Chollet et al., 2004).

The different structural features of tested compounds allow showing how the nature and the position of their diverse molecular fragments modulate on the one hand their selectivity regarding AcrAB pump and on the other hand their chemosensitizing activity. The tested compounds exhibiting the common hydantoin scaffold were modified using two of diverse substituents at position 5 as well as amine substituents and a selection of linkers to bind an amine to the hydantoin core. The location of an amine-alkyl fragment at position N1 or N3 to an aryl fragment at position 5 was modified for comparing with the activity of the first generations of hydantoin synthesized (Handzlik et al., 2011). In the case of **32**, the hydrophilic primary amine fragment is focused by terminate location at the end of longer 3-hydantoin substitution, opposite to the 5-β-nahthyl one.

The most active compounds (**29–32**) share the same pharmacophore profile than PAβN suggesting a similar physicochemical outline for an identical target. The amphiphilic nature of the Generation IIIA of optimized derivatives seems to be crucial to inhibit antibiotic resistance mediated by efflux pump and open new ways for generating original active compounds able to inhibit pump activity. With the recent published data regarding piperazine arylideneimidazolone derivatives as potential efflux inhibitors in *Escherichia coli* cells (Bohnert et al., 2016), the compound **32** characterized in this study will be used for pharmacophoric modulations in order to develop more potent inhibitors.

AUTHOR CONTRIBUTIONS

JH, KK-K, J-MP, and SA designed research; EO-M, JC, ES, and SA performed research; JS, GB, and J-MB contributed new

reagents or analytic tools; EO-M, JH, KK-K, J-MP, and SA analyzed data; JH, KK-K, J-MP, and SA wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00622

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