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Research review paper

Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that is a leading cause of morbidity and mortality in cystic fibrosis patients and immunocompromised individuals. Eradication of *P. aeruginosa* has become increasingly difficult due to its remarkable capacity to resist antibiotics. Strains of *Pseudomonas aeruginosa* are known to utilize their high levels of intrinsic and acquired resistance mechanisms to counter most antibiotics. In addition, adaptive antibiotic resistance of *P. aeruginosa* is a recently characterized mechanism, which includes biofilm-mediated resistance and formation of multidrug-tolerant persister cells, and is responsible for recalcitrance and relapse of infections. The discovery and development of alternative therapeutic strategies that present novel avenues against *P. aeruginosa* infections are increasingly demanded and gaining more and more attention. Although mostly at the preclinical stages, many recent studies have reported several innovative therapeutic technologies that have demonstrated pronounced effectiveness in fighting against drug-resistant *P. aeruginosa* strains. This review highlights the mechanisms of antibiotic resistance in *P. aeruginosa* and discusses the current state of some novel therapeutic approaches for treatment of *P. aeruginosa* infections that can be further explored in clinical practice.

1. Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium belonging to the family Pseudomonadaceae that is able to survive in a wide range of environments (Silby et al., 2011). The genome of *P. aeruginosa* (5.5–7 Mbp) is relatively large compared to other sequenced bacteria such as *Bacillus subtilis* (4.2 Mbp), *Escherichia coli* (4.6 Mbp) and *Mycobacterium tuberculosis* (4.4 Mbp), and encodes a large proportion of regulatory enzymes important for metabolism, transportation and efflux of organic compounds. This enhanced coding capability of the *P. aeruginosa* genome allows for great metabolic versatility and high adaptability to environmental changes (Klockgether et al., 2011; Stover et al., 2000). *Pseudomonas aeruginosa* has been recognized as an opportunistic pathogen that is the most common bacterium associated with nosocomial infections and ventilator-associated pneumonia (Barbier et al., 2013). It rarely affects healthy individuals, but causes high morbidity and mortality in cystic fibrosis (CF) patients and immunocompromised individuals (Sadikot et al., 2005).

CF is a genetic disorder that is caused by mutations in both copies of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR), leading to formation of a thick mucus layer on the airway surfaces of CF patients, which hinders mucociliary clearance, reduces bacterial internalization by lung epithelial cells and inhibits antimicrobial peptides (Davies, 2002; Davies et al., 2007). Thus, the lung of a CF patient is a favourable environment for bacterial growth and colonization. *Pseudomonas aeruginosa* is the predominant pathogen causing CF lung infections; chronic infection with *P. aeruginosa* is recalcitrant to antibiotic treatment and results in declined pulmonary functions and ultimately to mortality in CF patients (Lyczak et al., 2002). In addition, *P. aeruginosa* accounts for over 5% of infectious exacerbations in patients with chronic obstructive pulmonary disease (COPD) and has been associated with increased mortality of these patients (Murphy, 2009).

Empirical antibiotic therapy for suspected cases of *P. aeruginosa* includes monotherapy and combination therapy; this therapy reduces the mortality in patients with severe *P. aeruginosa* infections (El Solh and Alhajhusain, 2009; Park et al., 2012). However, treatment of *P. aeruginosa* infections has become a great challenge due to the ability of

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this bacterium to resist many of the currently available antibiotics (Lister et al., 2009). The World Health Organization (WHO) has recently listed carbapenem-resistant *P. aeruginosa* as one of three bacterial species in which there is a critical need for the development of new antibiotics to treat infections (Tacconelli et al., 2017). Moreover, excessive use of antibiotics during treatment accelerates development of multidrug-resistant *P. aeruginosa* strains, leading to the ineffectiveness of the empirical antibiotic therapy against this microorganism (Hirsch and Tam, 2010).

Pseudomonas aeruginosa displays resistance to a variety of antibiotics, including aminoglycosides, quinolones and β-lactams (Hancock and Speert, 2000). Generally, the major mechanisms of *P. aeruginosa* used to counter antibiotic attack can be classified into intrinsic, acquired and adaptive resistance. The intrinsic resistance of *P. aeruginosa* includes low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic-inactivating enzymes. The acquired resistance of *P. aeruginosa* can be achieved by either horizontal transfer of resistance genes or mutational changes (Breidenstein et al., 2011). The adaptive resistance of *P. aeruginosa* involves formation of biofilm in the lungs of infected patients where the biofilm serves as a diffusion barrier to limit antibiotic access to the bacterial cells (Drenkard, 2003). In addition, multidrug-tolerant persister cells that are able to survive antibiotic attack can form in the biofilm; these cells are responsible for prolonged and recurrent infections in CF patients (Mulcahy et al., 2010). Development of new antibiotics or alternative therapeutic strategies for treatment of *P. aeruginosa* infections is urgently required for the patients whose infections are resistant to conventional antibiotics. New antibiotics with novel modes of action have been explored in recent years, as have new routes of administration and resistance to modification by bacterial enzymes. Some of these newer antibiotics show excellent *in vitro* antibacterial activity against *P. aeruginosa* as well as lower minimum inhibitory concentration (MIC) compared to conventional antibiotics (Cigana et al., 2016; El Solh and Alhajhusain, 2009; Walkty et al., 2014). In addition, recent studies have reported several novel non-antibiotic therapeutic approaches that are highly effective in killing antibiotic-resistant *P. aeruginosa* strains. These approaches include: inhibition of quorum sensing and bacterial lectins, use of iron chelation, phage therapy, vaccine strategy, nanoparticles, antimicrobial peptides and electrochemical scaffolds. These therapeutic approaches can be used as either an alternative to or in combination with conventional antibiotic treatments (Chatterjee et al., 2016). This review summarizes the recent findings regarding mechanisms of intrinsic, acquired and adaptive antibiotic resistance in *P. aeruginosa* and describes several new antibiotics and novel therapeutic strategies to combat *P. aeruginosa* infections.

2. Intrinsic antibiotic resistance

The intrinsic antibiotic resistance of a bacterial species refers to its innate ability to diminish the efficacy of a specific antibiotic through inherent structural or functional characteristics (Blair et al., 2015). *Pseudomonas aeruginosa* has been shown to possess a high level of intrinsic resistance to most antibiotics through restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell and production of antibiotic-inactivating enzymes such as β-lactamases (Fig. 1) (Breidenstein et al., 2011).

2.1. Outer membrane permeability

Most antibiotics used to treat *P. aeruginosa* infections must be able to penetrate the cell membrane to reach intracellular targets (Lambert, 2002). For example, the aminoglycoside family of antibiotics such as tobramycin, gentamicin, and amikacin inhibits bacterial protein synthesis by binding to ribosomal 30S subunits (Mingeot-Leclercq et al., 1999). Quinolone antibiotics such as ciprofloxacin and levofloxacin interfere with DNA replication by inhibiting DNA gyrase and topoisomerase IV (Aldred et al., 2014). The β-lactam antibiotics including penicillin, cephalosporin, carbapenem and monobactam contain a β-lactam ring in their molecular structures. This class of antibiotics blocks bacterial cell wall biosynthesis by targeting the penicillin-binding proteins that are enzymes involved in peptidoglycan synthesis (Poole, 2004). Polymyxins are a group of polypeptide antibiotics that bind to the lipopolysaccharides (LPS) on the outer membrane of Gram-negative bacteria, leading to increased cell membrane permeability and enhanced antibiotic uptake. Polymyxin B and polymyxin E, also known as colistin, are the two polymyxins used in clinical practice, and they kill bacteria by induction of a hydroxyl radical-mediated cell death pathway (Zavascki et al., 2007). To enter the bacterial cell, β-lactams and quinolones penetrate cell membranes through porin channels, whereas aminoglycosides and polymyxins promote their own uptake by interacting with bacterial LPS on the outer membrane of Gram-negative bacteria (Lambert, 2002). Examples and chemical structures of aminoglycoside, quinolone, β-lactam and polymyxin antibiotics are summarized in Table 1.

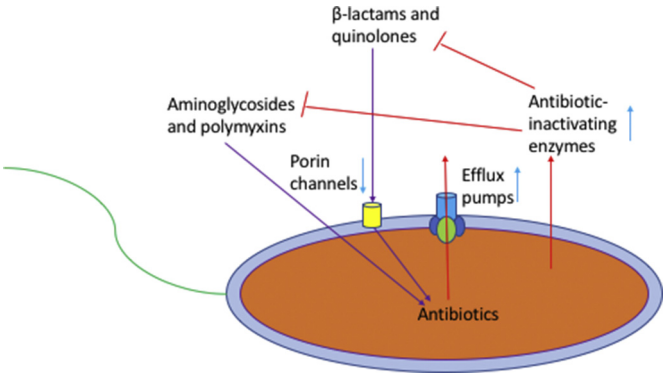
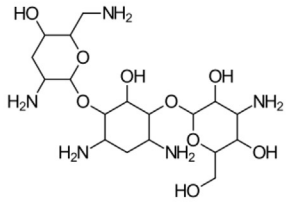
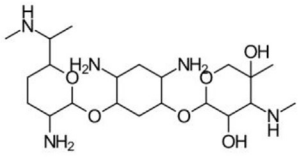
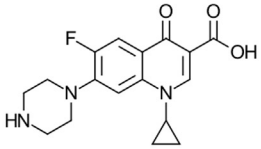
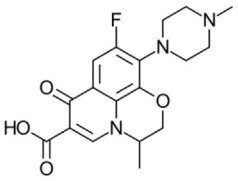
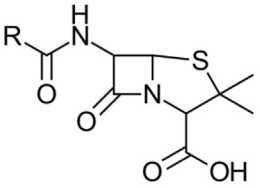
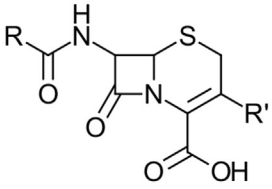
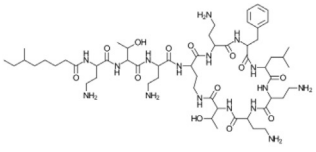
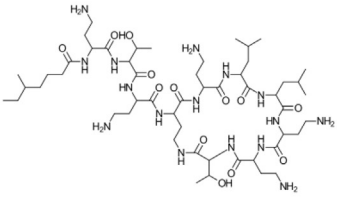


Fig. 1. A schematic representation of the mechanisms of intrinsic antibiotic resistance in *P. aeruginosa*. The mechanisms of intrinsic antibiotic resistance possessed by *P. aeruginosa* include restricted outer-membrane permeability, efflux systems that pump antibiotics out of the cells and production of antibiotic-inactivating enzymes. Quinolones and β-lactams penetrate cell membranes through porin channels. Aminoglycosides and polymyxins promote their own uptake by interacting with *P. aeruginosa* LPS on the outer membrane.

The outer membrane of Gram-negative bacteria, such as *P. aeruginosa*, which acts as a selective barrier to prevent antibiotic penetration, is an asymmetric bilayer of phospholipid and LPS, embedded with porins that form β-barrel protein channels (Delcour, 2009). Generally, the family of porins can be divided into four classes: the non-specific porins, which allow for slow diffusion of most of the small hydrophilic molecules; specific porins, which possess specific sites to bind a particular set of molecules; gated porins, which are ion-regulated outer membrane proteins responsible for uptake of ion complexes; and efflux porins, which are important components of efflux pumps (Hancock and Brinkman, 2002; Welte et al., 1995). In *P. aeruginosa*, the OprF protein is the major non-specific porin; OprB, OprD, OprE, OprO and OprP are specific porins; and OprC and OprH belong to the class of gated porins. The class of efflux porins include OprM, OprN and OprJ (Hancock and Brinkman, 2002).

The outer membrane permeability of *P. aeruginosa* is extremely restricted; it is about 12- to 100-fold lower than that of *E. coli* (Bellido et al., 1992; Hancock and Brinkman, 2002). OprF, a homolog of *E. coli* outer membrane protein A (OmpA), is the predominant porin of *P. aeruginosa* and is responsible for non-specific uptake of ions and saccharides including trisaccharides and tetrasaccharides, but it has low efficiency for antibiotic permeation (Bellido et al., 1992; Nikaido et al., 1991). OprF is able to fold into two conformers: the two-domain closed conformer consisting of an N-terminal transmembrane β-barrel and a C-terminal periplasmic globular domain, and the one-domain open-channel conformer containing a single transmembrane domain. The

Table 1
Examples and chemical structures of aminoglycoside, quinolone, β-lactam and polymyxin antibiotics.

Antibiotics used to treat *P. aeruginosa*	Examples and Chemical Structures	
Aminoglycoside	 Tobramycin	 Gentamicin
Quinolone	 Ciprofloxacin	 Levofloxacin
β-lactam	 Penicillin	 Cephalosporin
Polymyxin	 Polymyxin B	 Polymyxin E (Colistin)

closed conformer is the dominant structure of OprF channels, and only a small fraction of OprF form open channels, representing less than 5% of this protein population (Sugawara et al., 2006). The presence of mostly closed OprF channels may explain why the outer membrane permeability of *P. aeruginosa* is much lower than other bacteria. Additionally, absence of the *P. aeruginosa* OprF leads to increased biofilm formation through upregulation of bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), which is an important messenger for controlling biofilm formation (Bouffartigues et al., 2015).

As mentioned above, *P. aeruginosa* possesses a number of specific porins, including the carbohydrate-specific porin OprB, the basic amino acid-specific porin OprD, the phosphate-specific porin OprP, and the pyrophosphate-specific porin OprO (Hancock and Brinkman, 2002). Among these porins, OprD is involved in antibiotic uptake. It contains the binding sites for carbapenems, a class of β-lactam antibiotics, and absence of OprD in *P. aeruginosa* increases the resistance to this class of antibiotic (Li et al., 2012). Additionally, OprH is the smallest *P. aeruginosa* porin, and overexpression of OprH as a consequence of Mg^{2+} starvation has been found to be associated with increased resistance to polymyxin B and gentamicin through stabilization of the outer membrane by inducing LPS modification (Bell et al., 1991; Macfarlane et al., 1999).

2.2. Efflux systems

Bacterial efflux pumps play an important role in expelling toxic compounds out of the cell, and can be classified into five families: resistance-nodulation-division (RND) family, major facilitator superfamily (MFS), ATP-binding cassette (ABC) superfamily, small multidrug resistance (SMR) family, and multidrug and toxic compound extrusion

(MATE) family (Sun et al., 2014). In particular, the proteins belonging to the RND family of efflux pumps play a key role in antibiotic resistance in *P. aeruginosa* (Li and Nikaido, 2009). They consist of cytoplasmic membrane transporters, periplasmic linker proteins and outer membrane porin channel proteins (Daury et al., 2016). The cytoplasmic and periplasmic components of *P. aeruginosa* RND pumps are named multidrug efflux (Mex) along with a letter, and the outer membrane porin is named Opr along with a letter. *Pseudomonas aeruginosa* expresses twelve RND family efflux pumps, four of which (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) contribute to antibiotic resistance (Dreier and Ruggerone, 2015). MexAB-OprM is responsible for efflux of β-lactams and quinolones (Dupont et al., 2005; Masuda et al. 2000). MexCD-OprJ is able to pump out β-lactams (Okamoto et al., 2002). MexEF-OprN is capable of extruding quinolones (Llanes et al., 2011), while MexXY-OprM expels aminoglycosides (Hocquet et al., 2003; Masuda et al., 2000). Overexpression of multiple efflux pumps has been found in some clinical strains of *P. aeruginosa*, broadening bacterial antibiotic resistance and contributing to the development of multidrug-resistance (Cabot et al., 2011; Llanes et al., 2004; Shigemura et al., 2015). Furthermore, the use of efflux pump inhibitors has emerged as a potential therapeutic strategy for treatment of *P. aeruginosa* infections (Askoura et al., 2011). Phenylalanine arginyl β-naphthylamide (PAβN) is a well-studied efflux pump inhibitor that not only impairs antibiotic efflux through competitive inhibition of efflux pumps but also increases the permeability of bacterial outer membranes (Lamers et al., 2013). This compound has been shown to reduce virulence, diminish quorum sensing and increase antibiotic susceptibility of *P. aeruginosa* (El-Shaer et al., 2016; Lamers et al., 2013; Rampioni et al., 2017).

2.3. Antibiotic-inactivating enzymes

Production of antibiotic-inactivating enzymes that break down or modify antibiotics is one of the major mechanisms of intrinsic resistance in bacteria. Many antibiotics have chemical bonds such as amides and esters that are susceptible to hydrolysis (Wright, 2005) by enzymes commonly produced by *P. aeruginosa* such as β-lactamases and aminoglycoside-modifying enzymes (Poole, 2005; Wolter and Lister, 2013).

Like other Gram-negative bacteria, *P. aeruginosa* possesses an inducible *ampC* gene, encoding the hydrolytic enzyme β-lactamase. This enzyme is able to break the amide bond of β-lactam ring, leading to inactivation of β-lactam antibiotics (Wright, 2005). Furthermore, β-lactamases can be divided into four classes, A, B, C and D, based on their amino acid sequences. The enzyme classes A, C, and D hydrolyze β-lactams through an active site serine. By contrast, the class B β-lactamases are metalloenzymes that require divalent zinc ions for β-lactam hydrolysis (Bush and Jacoby, 2010). The class C β-lactamase produced by *P. aeruginosa* has been shown to inhibit antipseudomonal cephalosporins, a class of β-lactams (Berrazeg et al., 2015). Some *P. aeruginosa* isolates have been found to produce extended-spectrum-β-lactamases (ESBLs) which confer a high degree of resistance to the majority of β-lactam antibiotics, including penicillins, cephalosporins and aztreonam (Paterson and Bonomo, 2005; Rawat and Nair, 2010). ESBLs are predominantly in enzyme class A, though the OXA-type ESBLs, which were named for their oxacillin-hydrolyzing abilities, are in enzyme class D and were first identified in *P. aeruginosa* isolates (Rawat and Nair, 2010). In order to overcome β-lactamase-mediated resistance, β-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam have been developed and applied in clinical practice, and found to greatly increase the efficacy of β-lactams in combination therapies (Drawz and Bonomo, 2010).

Aminoglycosides, which contain an aminocyclitol ring linked to amino sugars by glycosidic bonds (Ratjen et al., 2009), are a group of antibiotics commonly used in treatment of *P. aeruginosa* infections. Aminoglycoside resistance in *P. aeruginosa* is due to multiple factors, such as reduced cell membrane permeability, increased efflux, ribosomal changes and enzyme modification. Among these mechanisms, the enzymatic modification of amino and glycoside groups in the aminoglycoside molecular structure plays a predominant role in resistance to this class of antibiotics (Ratjen et al., 2009). Three types of aminoglycoside-modifying enzymes have been discovered in bacteria: aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferase (AAC) and aminoglycoside nucleotidyltransferase (ANT) (Ramirez and Tolmasky, 2010). *Pseudomonas aeruginosa* APHs have been found to transfer a phosphoryl group to the 3′-hydroxyl of aminoglycosides such as kanamycin, neomycin and streptomycin, thereby inactivating these antibiotics (Hachler et al., 1996; Hainrichson et al., 2007; Poole, 2005). The AACs of *P. aeruginosa* have been found to transfer an acetyl group to the amino group at position 3′ and 6′ of aminoglycosides, which is responsible for the inactivation of gentamicin, tobramycin, netilmicin, kanamycin and amikacin (Poole, 2005). Resistance to gentamicin, amikacin and tobramycin is conferred by the ANTs of *P. aeruginosa*, which transfer an adenylyl group to either the amino or hydroxyl group of these aminoglycosides (Jacoby et al., 1990; Subedi et al., 2018).

3. Acquired antibiotic resistance

Bacteria can gain antibiotic resistance through mutational changes or acquisition of resistance genes via horizontal gene transfer (Munita and Arias, 2016). In addition to the high level of intrinsic antibiotic resistance of *P. aeruginosa*, the acquired resistance greatly contributes to development of multidrug-resistant strains, which increases the difficulty in eradicating this microorganism and leads to more cases of persistent infections (Henrichfreise et al., 2007).

3.1. Resistance by mutations

Mutational changes are able to cause reduced antibiotic uptake, modifications of antibiotic targets, and overexpression of efflux pumps and antibiotic-inactivating enzymes; all of which allow bacteria to survive in the presence of antimicrobial molecules (Munita and Arias, 2016). For example, a study by Mandsberg et al. (2009) demonstrated that inactivation of the DNA oxidative repair system increases mutation frequencies in *P. aeruginosa* leading to enhanced β-lactamase production and overexpression of the MexCD-OprJ efflux pump.

Porins form small water-filled channels within membranes that mediate the diffusion of hydrophilic antibiotics, up to a certain size exclusion limit (Welte et al., 1995). Spontaneous mutations can affect the expression or function of a specific porin, thereby reducing bacterial membrane permeability and increasing antibiotic resistance (Fernandez and Hancock, 2012). For instance, a deficiency in OprD in *P. aeruginosa* confers a high level of resistance to carbapenems, especially to imipenem (Fang et al., 2014; Li et al., 2012; Wolter et al., 2004). Fang et al., (2014) analyzed 61 imipenem-resistant *P. aeruginosa* clinical isolates from southern China, and they found that 50 isolates had mutations that resulted in disrupted OprD by either frameshift mutations or a premature stop codon, and 5 isolates had reduced *OprD* expression, whereas *OprD* was not detectable by PCR in 6 isolates. Furthermore, a functional study revealed that loops 2 and 3 in the OprD protein contained the entrance and/or binding sites for imipenem. Thus, mutations in loops 2 and/or 3 of OprD caused conformational changes and induced carbapenem resistance (Ochs et al., 2000).

As mentioned earlier, to prevent the intracellular accumulation of toxic compounds, bacteria employ energy-dependent efflux systems to pump the toxic molecules out of the cells (Sun et al., 2014). Thus, *P. aeruginosa* clinical isolates with overexpressed efflux pumps have decreased susceptibility to antibiotics (Cabot et al., 2011; Cabot et al., 2016; Llanes et al., 2004; Poonsuk et al., 2014). For example, overexpression of *P. aeruginosa* MexAB-OprM, which occurred as a consequence of gene mutations of transcriptional regulators, *mexR*, *nalB*,

nalC or *nalD*, enhanced resistance of the bacterium to β-lactams and fluoroquinolones, a class of quinolone (Braz et al., 2016; Saito et al., 1999; Srikumar et al., 2000; Tian et al., 2016). Overexpression of MexXY–OprM induced by *mexZ* gene mutation led to increased resistance to aminoglycoside, β-lactam and fluoroquinolone antibiotics in clinical isolates of *P. aeruginosa* (Baum et al., 2009; Guenard et al., 2014; Hocquet et al., 2006). The *P. aeruginosa* strains with mutations in the *nfxB* gene that encodes a transcriptional regulator, have overexpressed MexCD–OprJ, and are less susceptible to fluoroquinolones and penem antibiotics, a β-lactam subfamily (Okamoto et al., 2002; Poole et al., 1996).

Interference with antibacterial targets is a common strategy that bacteria utilize to avoid the antimicrobial action of antibiotics, and it can be achieved through protection of the targets and modifications of the target sites (Munita and Arias, 2016). Thus, mutational modifications of the target sites in *P. aeruginosa* also contribute to its antibiotic resistance. One of the best characterized examples is modification of the quinolone target sites. As mentioned above, quinolone antibiotics inhibit bacterial DNA replication by targeting DNA gyrase and topoisomerase IV (Aldred et al., 2014) so that mutations in genes encoding DNA gyrase (*gyrA* and *gyrB*) and/or topoisomerase IV (*parC* and *parE*) cause a decrease of the binding affinity of the encoded proteins to quinolones, leading to reduced susceptibility to quinolones in *P. aeruginosa* (Bruchmann et al., 2013). *Pseudomonas aeruginosa* strains with ribosomal mutations have been shown to develop a high level of resistance to aminoglycosides, since this antibiotic group inhibits protein translation by targeting the 30S ribosomal subunit. (El'Garch et al., 2007). Modification of penicillin-binding proteins in *P. aeruginosa* has been reported to increase resistance to β-lactam antibiotics (Moya et al., 2012). Polymyxin resistance in *P. aeruginosa* was shown to associate with modification of the polymyxin-binding partner LPS by addition of 4-amino-L-arabinose (L-Ara4N) to the phosphate groups within the lipid A moiety of LPS (Boll et al., 1994). Additionally, mutations in the two-component regulatory systems of PhoPQ and PmrAB promoted modification of aminoarabinose addition to lipid A, leading to enhanced polymyxin resistance in *P. aeruginosa* (Miller et al., 2011; Moskowitz et al., 2004; Owusu-Anim and Kwon, 2012).

Mutation causing overexpression of antibiotic-inactivating enzymes in *P. aeruginosa* is another well-characterized mechanism of acquired resistance (Munita and Arias, 2016). Some *P. aeruginosa* clinical isolates have overproduction of β-lactamases caused by mutations in a β-lactamase inducible gene *ampC*, which greatly increased the resistance to cephalosporins (Berrazeg et al., 2015). Moreover, inactivating mutations in the *ampD* gene, which encodes a cytosolic N-acetyl-anhydromuramil-l-alanine amidase and acts as a repressor of *ampC* expression, resulted in hyperproduction of β-lactamases in *P. aeruginosa* (Juan et al., 2005).

3.2. Acquisition of resistance genes

Antibiotic resistance genes can be carried on plasmids, transposons, integrons and prophages, and bacteria can acquire these genes via horizontal gene transfer from the same or different bacterial species (Breidenstein et al., 2011). Integrons are genetic elements that insert mobile gene cassettes into a specific genetic site via site-specific recombination (Hall and Collis, 1995), and they have been shown to play a critical role in dissemination of antibiotic resistance among *P. aeruginosa* strains (Chen et al., 2009; Khosravi et al., 2017; Nikokar et al., 2013; Odumosu et al., 2013). The main mechanisms of horizontal gene transfer involve transformation, transduction and conjugation (Fig. 2) (Arber, 2014). Acquisition of aminoglycoside and β-lactam resistance genes has been reported in *P. aeruginosa* (Bonomo and Szabo, 2006; Cavalcanti et al., 2015; Hong et al., 2015; Poole, 2011; Yan et al., 2006). For example, six types of *P. aeruginosa* metallo-beta-lactamases (MBLs), which belong to class B β-lactamases that hydrolyze most β-lactam-based antibiotics, have been described, including imipenemase

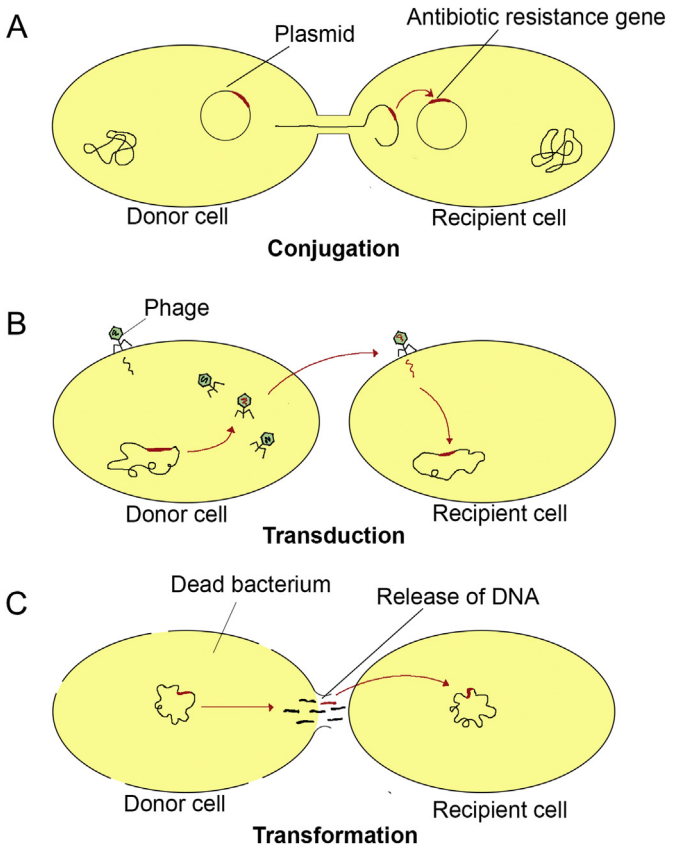


Fig. 2. Mechanisms of horizontal gene transfer. Bacterial DNA can be transferred from one bacterium to another via horizontal gene transfer. The horizontal transfer mechanisms include conjugation, transduction and transformation. (A) Conjugation is a process that transfers DNA through direct physical contact between the donor cell and the recipient cell. (B) Transduction is the transfer of DNA from one bacterium to another by bacteriophages. (C) In transformation, bacteria take up free fragments of DNA released into the environment and incorporate it into their own genome.

(IMP), Verona integron-encoded metallo-β-lactamase (VIM), Sao Paulo metallo-β-lactamase (SPM), Germany imipenemase (GIM), New Delhi metallo-β-lactamase (NDM) and Florence imipenemase (FIM) (Hong et al., 2015). The genes for these *P. aeruginosa* MBLs have been detected being carried by genetic elements, including integrons and plasmids (Bonomo and Szabo, 2006; Castanheira et al., 2004; Cavalcanti et al., 2015; Khajuria et al., 2013; Yan et al., 2006). Additionally, multiple antibiotic resistance genes can be carried in a single integron. Poirel et al. (2001) identified two novel aminoglycoside resistance genes, *aacA29a* and *aacA29b*, which are located at the 5′ and 3′ end of the carbapenem-hydrolyzing β-lactamase VIM-2 gene cassette, respectively, in class I integrons of *P. aeruginosa* clinical isolates.

4. Adaptive antibiotic resistance

Adaptive resistance increases the ability of a bacterium to survive antibiotic attack due to transient alterations in gene and/or protein expression in response to an environmental stimulus, and it is reversible when the stimulus is removed (Sandoval-Motta and Aldana, 2016). In *P. aeruginosa*, the best characterized mechanisms of adaptive resistance are the formation of biofilm and the generation of persister cells, which result in persistent infection and poor prognosis in CF patients (Taylor et al., 2014).

4.1. Biofilm-mediated resistance

A biofilm is an aggregate of microorganisms that adhere to each other on a living or non-living surface, and are embedded within a self-produced matrix of extracellular polymeric substances (EPSs), including exopolysaccharides, proteins, metabolites and extracellular DNA (eDNA) (Das et al., 2013; Donlan, 2002). The microbial cells grown in biofilms are less sensitive to antimicrobial agents and host immune response than the cells grown in free aqueous suspension (Stewart and Costerton, 2001). Even bacteria that are deficient in intrinsic resistance or lack protective mutations, can become less susceptible to antibiotics when they grow in a biofilm (Stewart, 2002). For example, a strain of *Klebsiella pneumoniae* with defective β-lactamase production grown in membrane-supported biofilms showed a higher minimum inhibitory concentration and a lower permeation of ampicillin and ciprofloxacin compared to the same strain grown in suspension culture (Anderl et al., 2000). Furthermore, the antibiotic sensitivity can be rapidly restored when bacteria lose biofilm protection, suggesting that the biofilm-mediated resistance is independent of genetic mutations, and it is an adaptive mechanism (Walters et al., 2003). The general mechanisms of biofilm-mediated resistance protecting bacteria from antibiotic attack involve prevention of antibiotic penetration, altered microenvironment inducing slow growth of biofilm cells, induction of an adaptive stress response and persister cell differentiation (Fig. 3) (Stewart, 2002).

Pseudomonas aeruginosa causes chronic infections in the lungs of CF patients, and forms biofilm on lung epithelial cell surfaces by production of DNA, proteins and exopolysaccharides (Taylor et al., 2014). Regulation of *P. aeruginosa* biofilm formation is multifactorial, and it mainly depends on quorum sensing systems, the two-component regulatory systems GacS/GacA and RetS/LadS, exopolysaccharides and c-di-GMP (Rasamiravaka et al., 2015). Quorum sensing is a bacterial cell-cell communication process that regulates gene expression in response to changes in cell-population density (Miller and Bassler, 2001). *Pseudomonas aeruginosa* possesses three main quorum sensing systems,LasI-LasR, RhlI-RhlR, and PQS-MvfR, all of which contribute to the formation of mature and differentiated biofilms (de Kievit and Iglewski, 2000; Kang et al., 2017; Storz et al., 2012). A GacA-deficient *P. aeruginosa* strain PA14 manifested a 10-fold reduction in biofilm formation capacity compared to wild-type PA14, suggesting a positive regulatory role of the GacS/GacA system in biofilm formation (Parkins et al., 2001). In contrast, the sensor kinase RetS in the RetS/LadS system repressed *P. aeruginosa* biofilm formation (Goodman et al., 2004). Exopolysaccharides produced by *P. aeruginosa* include alginate, Pel and Psl, and these stabilize the biofilm structure. The *P. aeruginosa* mutants deficient in biosynthesis of alginate, Pel and/or Psl lost their ability to form biofilms (Ghafoor et al., 2011). Additionally, eDNA released by lysed cells is another important component of the biofilm matrix, which facilitates initial cell-cell adhesion and aggregation on surfaces (Das et al., 2010). A novel function of the eDNA of *P. aeruginosa* was identified, as the eDNA was shown to acidify the environment and induce the expression of genes regulated by the PhoPQ and PmrAB two-component regulatory systems, leading to greatly increased aminoglycoside resistance (Wilton et al., 2016). The small intracellular molecule c-di-GMP is a nucleotide second messenger in signaling transduction in bacteria (Hengge 2009), and high intracellular levels of c-di-GMP are associated with biofilm formation, whereas low c-di-GMP levels are associated with planktonic type of cells (Ha and O'Toole, 2015). Furthermore, c-di-GMP has been found to regulate the processes that are important for cell-cell attachment and exopolysaccharide production, which contribute to formation and maturation of *P. aeruginosa* biofilm (Ha and O'Toole, 2015).

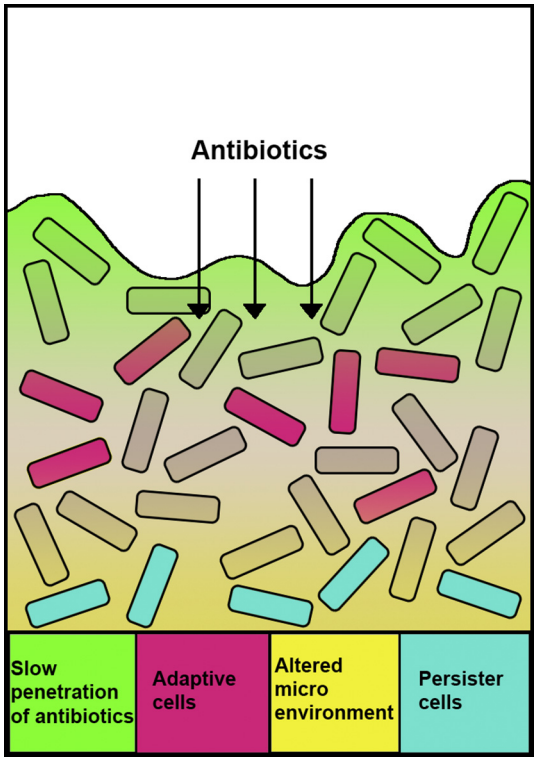


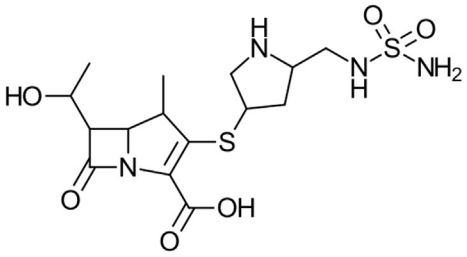
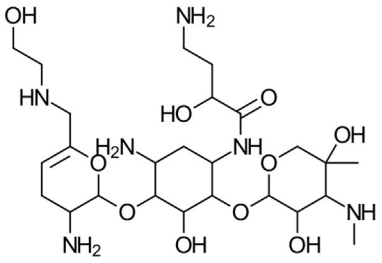
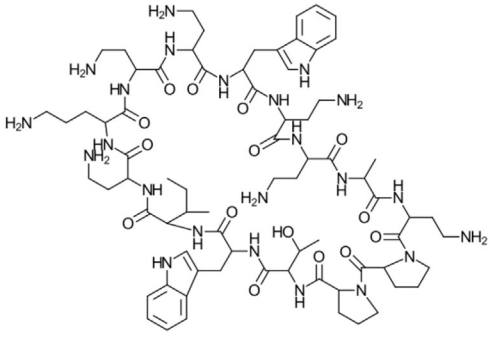
Fig. 3. Mechanisms of biofilm-mediated antibiotic resistance. Antibiotics slowly penetrate the biofilm (green); Some biofilm cells express an adaptive stress response permitting survival under harsh conditions (pink); The altered chemical microenvironment (yellow) within the biofilm induces slow growth of bacteria, which reduces antibiotic uptake; Multidrug-tolerant persister cells are formed (blue).

Pseudomonas aeruginosa undergoes numerous physiological and phenotypic changes during biofilm formation (Drenkard 2003). For example, in CF chronic infection, *P. aeruginosa* strains convert to a mucoid phenotype that displays upregulated alginate production driven by the CF microenvironment, allowing for formation of biofilm colonies (Pritt et al., 2007). The *P. aeruginosa* flagellum is critical for initiation of biofilm formation due to its capability to exhibit swarming and twitching motility (O'Toole and Kolter, 1998). However, after surface attachment, *P. aeruginosa* significantly downregulates flagellum expression and may also permanently lose the flagellum due to genetic mutations, reducing activation of the host immune response, thereby allowing *P. aeruginosa* to evade immune detection and phagocytosis (Jyot et al., 2007). Furthermore, a study by Sadovskaya et al. (2010) identified a family of cyclic glycerophosphorylated β-(1, 3)-glucans secreted by *P. aeruginosa* to the extracellular biofilm matrix, that interacted with and sequestered kanamycin. A research group from the University of Ottawa identified several novel genes including *ndvB*, PA14_40260-40230 and *tssC1,* in the *P. aeruginosa* clinical isolate PA14, which did not affect biofilm formation but had an impact on biofilm-specific antibiotic resistance (Mah et al., 2003; Zhang et al., 2011; Zhang and Mah, 2008). Specifically, the *ndvB* gene encoded a glucosyltransferase important for the synthesis of periplasmic cyclic-β-(1, 3)-glucans that physically interacted with tobramycin and sequestered it in the periplasm before reaching its site of action (Mah et al., 2003). Gene PA14_40260-40230 is part of an operon that encodes a novel efflux pump, and deletion of this operon in *P. aeruginosa* resulted in a decrease of the resistance of the bacterium to gentamicin and ciprofloxacin in biofilm (Zhang and Mah, 2008). The *tssC1* gene is involved in type VI secretion of *P. aeruginosa* and is highly expressed in biofilm. Deletion of *tssC1* in *P. aeruginosa* caused a reduction in resistance to tobramycin, gentamicin, and ciprofloxacin, antibiotics that are commonly used in treatment of *P. aeruginosa* infection in CF patients (Zhang et al., 2011).

4.2. Persister cells in antibiotic resistance

Another major obstacle for treatment of *P. aeruginosa* infections is

Table 2
Chemical structures of doripenem, plazomicin and POL7001.

New antibiotics used to treat *P. aeruginosa*	Chemical Structures
Doripenem	
Plazomicin	
POL7001	

the formation of bacterial persister cells, phenotypic variants that are not genetically resistant to antibiotics but are tolerant to high concentrations of antibiotics. Persistence in the presence of antibiotics is a transient phenotype that is developed as a consequence of heterogeneous responses to the environment within a genetically identical bacterial population (Balaban et al., 2013). The observation that the formation of *P. aeruginosa* persisters and biofilms are interrelated both *in vitro* and *in vivo* makes the treatment of *P. aeruginosa* infections in CF lungs even more complicated (Drenkard and Ausubel, 2002). Persister cells comprise about 1% of biofilm cells, and are slow-growing, metabolically inactive and highly tolerant to antibiotics (Lewis, 2010; Wood et al., 2013). The majority of *P. aeruginosa* cells can be killed by antibiotics; however, persisters are able to remain viable and repopulate biofilms due to the existence of a dormant state that shuts down the synthesis of the antibiotic targets (Lewis, 2010; Van den Bergh et al., 2017). Persister cells do not proliferate in the presence of antibiotics, however, they resume growth once the antibiotics are removed (Maisonneuve and Gerdes, 2014). Thus, the remaining persister cells in biofilms are believed to be responsible for the recalcitrance of chronic infections. Toxin-antitoxin (TA) pairs are primarily responsible for formation of persister cells, which induce a state of dormancy (Wang and Wood, 2011). The TA systems consist of a protein toxin that disrupts an essential cellular process and an antitoxin, which can be either a protein or small non-coding RNA that inhibits the toxicity of the toxin. Moreover, the TA systems are involved in the regulation of a broad range of cellular processes including DNA replication, protein translation, plasmid maintenance and cell wall synthesis in response to various environmental stimuli (Unterholzner et al., 2013; Wen et al., 2014). The level of toxins and degradation of antitoxins are associated with persister formation. The first TA system related to persister cell formation was found to be the MqsR(toxin)/MqsA(antitoxin) pair in *E. coli*. In this system, deletion of the *mqsRA* locus decreased persister formation, whereas overexpression of MqsR increased persister cell formation (Kim and Wood, 2010). Other than MqsR, the toxins, TisB and HipA, have also been found to play a role in formation of *E. coli* persisters (Dorr et al., 2010; Kaspy et al., 2013). The precise nature of *P. aeruginosa* TA system-mediated persister cell formation is currently not clear, and it is still under investigation.

Pseudomonas aeruginosa strains isolated from CF patients typically have high levels of persister cells compared to wild-type strains (Mulcahy et al., 2010), making these *P. aeruginosa* cultures highly resistant to antibiotics with the potential to become multidrug-tolerant (Mlynarcik and Kolar, 2017). Furthermore, environmental stimuli have an impact on the formation and proliferation of persister cells. Nutrient deprivation enhances the formation of *P. aeruginosa* persister cells through the regulatory mechanism known as the stringent response that is mediated by the bacterial signaling molecule alarmone (p)ppGpp (Mlynarcik and Kolar, 2017; Nguyen et al., 2011). Moreover, the phenazine pyocyanin and the quorum-sensing signaling molecule acyl-homoserine lactone 3-OC12-HSL have been reported to greatly increase the number of persister cells in *P. aeruginosa* cultures (Moker et al., 2010). In addition, a study by Grassi et al. (2017) reported that exposure of stationary-phase *P. aeruginosa* cultures to cyanide m-chlorophenylhydrazone (CCCP), an uncoupling agent that reduces bacterial metabolic activity through inhibition of ATP production, was able to induce generation of persister cells with antibiotic-tolerant phenotypes.

5. New antipseudomonal antibiotics

Conventional antibiotic therapies against *P. aeruginosa* infections have become increasingly ineffective due to the rise of multidrug-resistant strains (Chatterjee et al., 2016). Current therapeutic options for *P. aeruginosa* treatment are the use of different antibiotic combinations and development of new antibiotics (Hancock and Speert, 2000). The new antibiotics have been shown to be more effective in *P. aeruginosa* killing and have a lower frequency of resistance development compared to existing antibiotics due to their novel modes of action, efficient drug delivery (e.g. inhaled antibiotics) and resistance to modification by bacterial enzymes (Chatterjee et al., 2016). The chemical structures of the new antibiotics, doripenem, plazomicin and POL7001 are presented in Table 2.

5.1. Doripenem

Doripenem is a new carbapenem antibiotic with broad spectrum activity against Gram-negative and Gram-positive bacteria through inhibition of bacterial cell wall synthesis by binding to penicillin-binding proteins; it has been approved by the US Food and Drug Administration (FDA) for treatment of complicated intra-abdominal infection and urinary tract infection (Greer, 2008; Paterson and Depestel, 2009). Doripenem is resistant to hydrolysis by many β-lactamases except for the class B metallo-β-lactamases (Queenan et al., 2010). Importantly, the *in vitro* antibacterial activity of doripenem against *P. aeruginosa* isolates from CF patients has been found to be more potent compared to other carbapenem antibiotics such as meropenem and imipenem (Castanheira et al., 2009; Riera et al., 2011; Traczewski and Brown, 2006). Furthermore, the efficacy of doripenem has been evaluated in patients with *P. aeruginosa* ventilator-associated pneumonia (Chastre et al., 2008; Luyt et al., 2014); a clinical phase III study of patients with *P. aeruginosa* ventilator-associated pneumonia found that the doripenem-treated patients had higher cure rates compared to the patients treated with imipenem (Chastre et al., 2008). Of note, the adverse effects of doripenem include headache, nausea, diarrhea, rash, and phlebitis (Hilas et al., 2008).

5.2. Plazomicin

Plazomicin is a next-generation, semisynthetic aminoglycoside antibiotic synthetically derived from the natural product sisomicin (Aggen et al., 2010). Plazomicin is able to resist a broad spectrum of aminoglycoside modifying enzymes, but not 16S rRNA ribosomal methyltransferases (Cox et al., 2018). Plazomicin demonstrates potent *in vitro* activity against both Gram-negative and Gram-positive bacterial pathogens, and it has similar activity to amikacin against multidrug-resistant *P. aeruginosa* strains (Walkty et al., 2014). Furthermore, Pankuch et al. (2011) reported an *in vitro* synergistic activity of plazomicin against *P. aeruginosa* clinical isolates when combined with cefepime, doripenem, imipenem or piperacillin-tazobactam, and no antagonism was observed in this study, suggesting that plazonmicin is a potential candidate for combination therapies in treatment of multidrug-resistant *P. aeruginosa* infections. Plazomicin may cause mild to moderate nephrotoxic and ototoxic effects (Karaiskos et al., 2015).

5.3. POL7001

Protein epitope mimetic (PEM) molecules have emerged as a novel class of antibiotics against *P. aeruginosa;* some PEM molecules inhibit the transport of LPS to the bacterial outer membrane (Srinivas et al., 2010). POL7001 is a macrocycle molecule belonging to the PEM antibiotic family. Cigana et al. (2016) evaluated the efficacy of POL7001 both *in vitro* and in murine models of *P. aeruginosa* acute and chronic pneumonia. They found that the multidrug-resistant *P. aeruginosa* isolates from CF patients were sensitive to POL7001, and that the POL7001-treated mice had a significantly reduced bacterial burden and decreased levels of inflammation in the lung during *P. aeruginosa* acute and chronic infection. The new mode of action, the efficient pulmonary delivery and the potent *in vitro* and *in vivo* activity suggest POL7001 as a novel therapeutic agent for future clinical trials. The side effects of POL7001 have not been reported yet.

6. Novel therapeutic strategies for *P. aeruginosa* treatment

The overuse and misuse of antibiotics is a growing concern for public health, which can result in unnecessary side effects and the development of drug-resistant bacterial strains (Ventola, 2015). Moreover, the development of new antibiotics is very limited and time-consuming. Thus, the development of novel therapeutic approaches to treat *P. aeruginosa* infections is highly desirable and has gained more attention in the past decade. These novel therapeutic strategies can act either alone or in combination with conventional therapies to combat *P. aeruginosa* infections, and they include inhibition of quorum sensing and bacterial lectins, as well as use of iron chelation, phage therapy, vaccine strategy, the use of nanoparticles, antimicrobial peptides and electrochemical scaffolds (Table 3) (Chatterjee et al., 2016; Hurley et al., 2012).

6.1. Quorum sensing inhibition

Quorum sensing is a mechanism that allows bacteria to control gene expression in a cell density-dependent manner (de Kievit and Iglewski, 2000). *Pseudomonas aeruginosa* utilizes quorum sensing to regulate virulence and biofilm formation (Christiaen et al., 2014). Las and Rhl are two major quorum-sensing systems of *P. aeruginosa*, that are responsible for the synthesis of the N-acyl homoserine lactone (AHL) signal molecules, N-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL), respectively (Glessner et al., 1999). The 3O-C12-HSL and C4-HSL bind to and activate their cognate transcription factors LasR and RhlR respectively, inducing biofilm formation and expression of various virulence factors including elastase, proteases, pyocyanin, lectins, rhamnolipids and toxins (Rutherford and Bassler, 2012). In addition to the LasI-LasR and RhlI-RhlR systems, the third *P. aeruginosa* quorum-sensing system PQS-MvfR has been reported to promote biofilm formation (Kang et al., 2017; Storz et al., 2012; Yang et al. 2009). This system controls production of the *Pseudomonas* quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone, through regulation of the *pqsABCD*E operon by the transcriptional regulator MvfR, also known as PqsR (Lee and Zhang, 2015). In addition, proteins PqsA and PqsD have been implicated in biofilm formation (Kang et al., 2017; Storz et al., 2012).

Inhibition of quorum sensing is considered a promising strategy for

Table 3
Summaries of alterative therapeutic strategies for treatment of *P. aeruginosa*

Therapeutic strategy	Advantages	Disadvantages	References
Quorum sensing inhibition	Prevention or reduction of biofilm formation, decrease of bacterial virulence, low risk of development of bacterial resistance	Narrow spectrum, unintentional effect on beneficial bacteria	Reuter et al., 2016, Rasmussen and Givskov, 2006
Lectin inhibition	High stability, low risk of development of bacterial resistance	Narrow spectrum due to expression of more than one type of adhesin by bacteria	Krachler and Orth, 2013, Ofek et al., 2003
Iron chelation	Availability of FDA-approved drugs, easy administration	Toxicity	Moreau-Marquis et al., 2009, Chitambar, 2010
Phage therapy	Replication at infection site, high specificity to target bacteria without effects on commensal flora, less side-effects, bactericidal activity against antibiotic-resistant bacteria, easy administration, delivery of antimicrobial agents to bacteria	Phage clearance after treatment, impurity of phage preparations, poor stability of phage preparations and lack of knowledge on the mode action of phages, bacterial resistance	Ly-Chatain, 2014, Sulakvelidze et al., 2001, Vandenheuvel et al., 2015
Vaccine strategy	Improvement of host immunity, prevention of infection	Low efficiency, no licensed vaccine	Priebe and Goldberg, 2014
Nanoparticles	High penetrability into bacterial membrane, disruption of biofilm formation, multiple antimicrobial mechanisms, good carriers of antibiotics	Toxicity	Wang et al., 2017, Elsaesser and Howard, 2012
Antimicrobial peptides	broad-spectrum activity, rapid killing kinetics, low levels of induced resistance, low toxicity to host	Hemolytic activity to host cells, reduced activity based on salt, serum, and pH sensitivity, susceptibility to proteolysis, high cost of production	Gordon et al., 2005, Hancock et al., 2016, Aoki and Ueda, 2013, Gordon et al., 2005
Electrochemical scaffold	Disruption of bacterial biofilms, increase of antibiotic penetration	Difficulty in implantation to clinical trials	Sultana et al., 2015

treatment of *P. aeruginosa* infections (Hurley et al., 2012). This approach is able to prevent or reduce biofilm formation, decrease bacterial virulence, and has a low risk of development of bacterial resistance (Reuter et al., 2016). Moreover, this approach has a narrow spectrum so that it is unlikely to have any unintended inhibitory effects on beneficial bacteria (Rasmussen and Givskov, 2006). The quorum sensing inhibitors for Las and Rhl systems can be either natural or synthetic, and are capable of reducing the activity of AHL synthase, inhibiting AHL production, degrading AHLs or competing for binding of AHL receptors (Kalia, 2013). The use of quorum sensing inhibitors for the treatment of *P. aeruginosa* infections has been intensively studied in recent years. For example, the carotenoid zeaxanthin, commonly found in plants, algae and lichens, reduced the biofilm formation in *P. aeruginosa* by binding to quorum sensing signal receptors, LasR and RhlR, and blocking expression of virulence genes, *lasB* and *rhlA* (Gokalsin et al., 2017). Flavonoids are a family of naturally produced plant metabolites that acted as antagonists of LasR and RhlR, and significantly reduced their binding ability to the promoters of quorum sensing-regulated genes in *P. aeruginosa* (Paczkowski et al., 2017). A synthetic quorum sensing inhibitor, N-decanoyl cyclopentylamide (C10-CPA), interfered with the Las and Rhl systems by inhibiting the binding of 3O-C12-HSL and C4-HSL to their cognate receptors, leading to impaired formation of *P. aeruginosa* biofilm and production of virulence factors, including elastase, pyocyanin and rhamnolipid (Ishida et al., 2007). Hentzer et al. (2002) introduced a synthetic compound, which is a derivative of halogenated furanone produced by the Australian macroalga *Delisea pulchra*, and found that it repressed quorum sensing-regulated virulence gene expression in *P. aeruginosa*. Moreover, they found that this synthetic halogenated furanone compound was able to penetrate *P. aeruginosa* microcolonies, disrupt the biofilm structure and induce bacterial detachment from a substratum surface.

MvfR has been suggested to be a potential target for inhibition of the PQS-MvfR quorum sensing system (Kitao et al., 2018; Maura and Rahme, 2017; Starkey et al. 2014). A benzamide-benzimidazole compound, M64, was used to chemically inhibit *P. aeruginosa* MvfR, leading to reduced biofilm formation and increased susceptibility of *P. aeruginosa* to meropenem and tobramycin (Maura and Rahme, 2017). Furthermore, it was found that M64 acted as a competitive antagonist of MvfR by binding to its ligand-binding domain, which reduced the DNA binding ability of MvfR (Kitao et al., 2018).

The macrolide antibiotic azithromycin is the only quorum sensing inhibitor that has been tested in clinical trials, and the beneficial effects of azithromycin treatment have been shown in CF patients with chronic pulmonary infections caused by *P. aeruginosa* (Imperi et al., 2014; Saiman et al., 2003; van Delden et al., 2012). Several studies have revealed that azithromycin significantly reduced the production of quorum sensing signal molecules and attenuated the virulence of *P. aeruginosa* (Bala et al., 2011; Tateda et al., 2001). However, it is noteworthy that the MexCD-OprJ efflux pump of *P. aeruginosa* confers resistance to azithromycin during biofilm formation (Gillis et al., 2005). Therefore, mutation-caused upregulated MexCD-OprJ expression may reduce the efficacy of azithromycin during treatment.

6.2. Lectin inhibition

Lectins are bacterial outer membrane proteins that recognize host glycoconjugates and allow bacteria to adhere to the host tissues (Nilsson, 2003). The adhesion of *P. aeruginosa* to lung epithelial cell surface is mediated by two specific lectins, LecA and LecB, that bind to the galactose and fucose surface receptors of the lung epithelial cells, respectively (Chemani et al., 2009). Furthermore, *P. aeruginosa* lectins have been found to be involved in biofilm formation by interacting with host cell glycoconjugates (Diggle et al., 2006; Tielker et al., 2005). Inhibition of lectin binding may be useful for prevention and treatment of *P. aeruginosa* infections for its high stability and low risk of development of bacterial resistance (Krachler and Orth, 2013). Lectin binding to host cell surfaces may be blocked by lectin inhibitors. These inhibitors, such as glycoclusters, glycopolymers and glycodendrimers, have high binding affinity for lectins and inhibit their functioning (Grishin et al., 2015). The β-phenylgalactosyl peptide dendrimer (GalAG2), a glycopeptide dendrimer, showed a strong binding affinity to *P. aeruginosa* LecA and inhibited biofilm formation *in vitro* (Kadam et al., 2011). Dendrimer FD2 binds to *P. aeruginosa* LecB, and facilitates the inhibition of biofilm formation and dispersion of pre-formed biofilms on a steel surface (Johansson et al., 2008). In addition to being chemically synthesized, the *P. aeruginosa* lectin inhibitors also occur naturally. Royal jelly is a secretion from young worker honeybees and contains nutrients for larvae development. It has been shown to inhibit *P. aeruginosa* biofilm formation by interacting with lectins and disrupting the initial attachment of *P. aeruginosa* to human lung epithelial cells *in vitro* (Lerrer et al., 2007; Susilowati et al., 2017). These *in vitro* studies showed that lectin inhibitors effectively inhibited *P. aeruginosa* biofilm formation, but they require further assessment in *in vivo* models and clinical trials. However, it is noteworthy that *P. aeruginosa*

expresses a variety of adhesins that can reduce the efficacy of lection inhibitors (Carnoy et al., 1994; Ofek et al., 2003).

6.3. Iron chelation

Iron is essential for bacterial growth and is involved in a variety of cellular processes, such as energy production, DNA replication and electron transport (Bullen et al., 1978; Ma et al., 2015). The iron content of human sputum was found to be significantly increased in CF patients compared to healthy individuals, suggesting that an increased amount of iron facilitates the chronic infection in CF lungs (Reid et al., 2007). *Pseudomonas aeruginosa* utilizes the siderophores pyoverdine and pyochelin to acquire iron from the extracellular environment (Cornelis and Dingemans, 2013). Thus, limiting the concentration of extracellular iron or disrupting iron uptake by *P. aeruginosa* is a strategy to counter *P. aeruginosa* infections. Numerous studies have linked iron metabolism to the pathogenesis of chronic infections and suggested that iron analogues and chelators may function as potential therapeutic agents against *P. aeruginosa*. For instance, it was reported that the iron chelators, 2,2′-dipyridyl (2DP), diethylenetriaminepentacetic acid (DTPA) and EDTA, impaired *P. aeruginosa* growth and biofilm formation, and they were more effective under anaerobic conditions (O'May et al., 2009). Gallium is a nonredox iron III analogue that disrupts bacterial iron metabolism by acting as an iron substitute in many biologic processes, consequently it is a US FDA-approved drug for treatment of cancer-associated hypercalcemia (Minandri et al., 2014). Kaneko et al. (2007) reported that gallium was able to inhibit *P. aeruginosa* growth, prevent biofilm formation, and manifest excellent bactericidal activity *in vitro* by decreasing bacterial iron uptake and repressing transcriptional regulator PvdS-mediated pyoverdine synthesis. Furthermore, gallium has also been found to effectively eradicate *P. aeruginosa* in mouse infection models (DeLeon et al., 2009; Kaneko et al., 2007). Unfortunately, patients receiving gallium compounds such as gallium nitrate and gallium arsenide for medical treatment may display immunosuppression and toxicity to various organs including kidney, lung and testis (Chitambar, 2010). However, iron chelators can be used alongside conventional antibiotics. In this regard, the combination of tobramycin with the iron chelators deferoxamine and deferasirox significantly reduced the biomass of *P. aeruginosa* biofilm on CF airway epithelial cells *in vitro* and enhanced the tobramycin-mediated killing of *P. aeruginosa* in biofilm (Moreau-Marquis et al., 2009). Since iron chelators such as deferoxamine, deferasirox and deferiprone are FDA-approved, they are considered to be relatively safe compared to other treatment options and should be considered in further clinical trials.

6.4. Phage therapy

Bacteriophages (phages) are viruses that infect and kill bacteria by causing lysis (Clokie et al., 2011). Phages were first discovered in 1915 by British bacteriologist Frederick Twort. Two years later, Félix d'Herelle in Paris independently made a similar finding and introduced the concept of phage therapy (Wittebole et al., 2014). Phage therapy has been practiced for decades in Eastern Europe, but has not gained wide acceptance in the Western world. There are several advantages of phage therapy, including replication at the infection site, high specificity to target bacteria without effects on commensal flora, fewer side-effects than other treatments, bactericidal activity against antibiotic-resistant bacteria and easy administration (Ly-Chatain, 2014). Use of phages for the treatment of *P. aeruginosa* infections has been extensively studied as an alternative to antibiotics. To date, there are 137 different phages targeting the *Pseudomonas* genus that have been characterized (Pires et al., 2015). Many *in vitro* and *in vivo* studies have been carried out to assess the efficiency of phages against *P. aeruginosa* chronic infections (Morello et al., 2011; Vieira et al., 2012; Waters et al., 2017). For example, it was demonstrated that co-incubation of phage PA709 with clinical strain *P. aeruginosa* 709 significantly reduced the viability of *P. aeruginosa* (Vieira et al., 2012). Another study showed that intranasal administration of bacteriophage P3-CHA to mice that received a lethal dose of *P. aeruginosa* strain CHA greatly increased the survival rate and decreased the bacterial burden in lungs (Morello et al., 2011). This study also found that pretreatment of mice with bacteriophage P3-CHA could effectively prevent *P. aeruginosa* CHA infection (Morello et al., 2011).

One of the advantages of phage therapy over antibiotic treatment is its activity against *P. aeruginosa* biofilms. A recent study revealed that phage PELP20 effectively killed *P. aeruginosa* strain LESB65 isolated from CF patients in an artificial sputum medium biofilm model *in vitro*, and greatly enhanced bacterial clearance in a mouse model of chronic lung infection with *P. aeruginosa* (Waters et al., 2017). Many microorganisms can develop biofilms on indwelling medical devices, such as catheters, and cause device-related infections (Singhai et al., 2012). Importantly, pretreatment of hydrogel-coated catheters with phage M4 significantly reduced *P. aeruginosa* biofilm formation (Fu et al., 2010). This finding suggests that pretreatment of the surface of indwelling medical devices with phage may be a potential benefit for CF patients, preventing *P. aeruginosa* infections during medical treatment. In addition, when a cocktail of four phages was used to treat *P. aeruginosa* biofilm *in vitro*, it was found that the phage cocktail significantly reduced the biofilm biomass, regardless of CF status or multidrug resistance (Fong et al., 2017).

Another advantage of phage therapy is that phages can be genetically engineered as vehicles to deliver antimicrobial agents to bacteria, thereby increasing the efficacy of treatment (Pires et al., 2016; Westwater et al., 2003). Nonlytic M13 phages were used to deliver the DNA sequences encoding the toxins Gef and ChpBK that induce bacterial cell death to *E. coli*, and this engineered phage showed effective bactericidal activity *in vitro* and in a mouse model of blood infection (Westwater et al., 2003). A variety of genetically engineered *E. coli* phages were constructed to degrade biofilms, impair bacterial DNA repair systems or target specific DNA sequences involved in antibiotic resistance and virulence by delivery of RNA-guided nucleases (Citorik et al., 2014; Lu and Collins, 2007; Lu and Collins, 2009). Moreover, it was suggested that some of the engineered phages may function as effective adjuvants for antibiotic therapy (Citorik et al., 2014, Lu and Collins, 2007, Lu and Collins, 2009). The use of genetically engineered *P. aeruginosa* phages has been tested over the past decade. The *P. aeruginosa* phage Pf3 was genetically modified to a nonlytic, non-replicating phage, Pf3R, by replacing an export protein gene with a BglII restriction endonuclease gene (Hagens et al., 2004). This modified phage efficiently killed *P. aeruginosa* PAO1 *in vitro,* similar to the lytic phage Pf3, but the engineered phage had largely reduced endotoxin release. Furthermore, as a consequence of the reduced endotoxin level and host inflammatory responses the Pf3R-treated mice displayed an increased survival rate compared to Pf3-treated mice (Hagens et al., 2004).

Although phages have been proven to be effective against bacterial infection *in vitro* and in animal models, only a limited number of clinical trials of phage therapy have been conducted to date. The reasons for this include: the safety concerns regarding phage clearance after treatment and impurity of phage preparations; poor stability of phage preparations; and lack of knowledge of the detailed mode action of phages and development of bacterial resistance to phages (Sulakvelidze et al., 2001; Vandenheuvel et al., 2015). The use of phages against *P. aeruginosa* infections in clinical trials has been examined in patients with venous leg ulcers, burn wounds and otitis, and no adverse effects were observed during these clinical trials (Merabishvili et al., 2009; Rhoads et al., 2009; Wright et al., 2009).

Bacterial resistance to phage is one of the major concerns, which may decrease the effectiveness of phage therapy (Sulakvelidze et al., 2001). The mechanisms of phage resistance in bacteria include: blocking phage adsorption to bacterial receptors; preventing phage DNA entry into the host cells by superinfection exclusion (Sie) systems;

cleaving phage genomic DNA by restriction–modification systems and Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR-associated (Cas) genes; and abortion of the phage infection by abortive infection (Abi) systems that target the crucial steps of phage multiplication (Labrie et al., 2010). *Pseudomonas aeruginosa* utilizes the mechanisms of phage adsorption blocking and the CRISPR-Cas system to counter phage attack (Cady et al., 2012; Harvey et al., 2018). For instance, *P. aeruginosa* was able to prevent pilus-specific phage infection through glycosylation of type IV pilins (Harvey et al., 2018). Bacteria and archaea use the CRISPR-Cas system to defend against bacteriophages and conjugative plasmids by cleaving the invading DNA (Marraffini and Sontheime,r 2010). When Cady et al. (2011) analyzed the CRISPR region in 122 *P. aeruginosa* clinical isolates, they found that 36% of the strains tested harbored CRISPR-Cas systems and the CRISPR spacers were 100% identical to prophages or sequenced temperate bacteriophages. Finally, they demonstrated that the *P. aeruginosa* isolates produced mature CRISPR RNAs (crRNAs), which suggests that the CRISPR and *cas* genes are expressed and functional in *P. aeruginosa*. In a separate study, Cady et al. (2012) provided the first evidence that *P. aeruginosa* utilizes the CRISPR/Cas system to mediate phage resistance. These findings suggest that phage therapy may be ineffective with CRISPR/Cas positive *P. aeruginosa* strains. Although phage-infected bacteria can become resistant to phages over time, the rate of developing resistance to phages is approximately 10-fold lower than to antibiotics (Sulakvelidze et al., 2001). Use of phage cocktails, rather than one type of phage, may be able to slow down the evolution of bacterial resistance to phages (Ormala and Jalasvuori, 2013). Moreover, phages remain one of largest genetic resources on earth, and they evolve rapidly to counter the resistance in bacteria (Stern and Sorek, 2011). In addition, it was shown that *P. aeruginosa* quorum sensing enhanced CRISPR-Cas expression and activity, and the quorum sensing inhibitor baicalein could suppress the CRISPR-Cas activity (Hoyland-Kroghsbo et al., 2017). Thus, the combination of quorum sensing inhibitors and phage therapy may be a promising therapeutic, approach that makes *P. aeruginosa* more susceptible to phages through inhibition of its CRISPR-Cas defense system.

6.5. Vaccine strategy

The idea of vaccine strategy is to prevent infection before it can become established. Development of vaccines aims to prevent and reduce *P. aeruginosa* infections. However, no licensed vaccine is as yet available against this pathogen (Priebe and Goldberg, 2014). *Pseudomonas aeruginosa* antigens elicit potent immune responses, and are responsible for pathogenesis (Lavoie et al., 2011). The potential candidates for *P. aeruginosa* vaccines are the LPS O-antigen, polysaccharide-protein conjugates, outer membrane proteins OprF and OprI, the type III secretion system component PcrV, flagella, pili, DNA, live-attenuated *P. aeruginosa* and whole killed cells (Doring and Pier, 2008). Among the possible *P. aeruginosa* vaccines, only the flagella vaccine and the recombinant vaccine IC43, comprising OprF and OprI, have proceeded to phase III clinical trials in CF patients (Doring et al., 2007, Vincent, 2014). The present vaccines for *P. aeruginosa* manifest low efficiency in clinical trials due to the ability of this pathogen to undergo phenotypic changes in variable environmental conditions (Doring and Pier, 2008). For example, the *P. aeruginosa* strains in the lungs of CF patients downregulate expression of highly immunogenic virulence factors, such as LPS O-antigen, type III secretion systems, flagella and pili (Gellatly and Hancock, 2013). Moreover, impaired host defense mechanisms also reduce the efficiency of vaccination (Grimwood et al., 2015). The lung microenvironment in CF patients has become a great challenge for successful vaccination due to the CF lungs having an altered mucus layer, impaired phagocytosis, and dysregulated inflammatory responses, including aberrant cytokine and chemokine production, and diminished phagocyte recruitment (Grimwood et al., 2015; Hartl et al., 2012). Therefore, the dysregulated innate immunity in CF lungs may affect the efficacy of *P. aeruginosa* vaccines. Development of novel *P. aeruginosa* vaccines is currently ongoing. For example, the protective efficacy of a novel *P. aeruginosa* vaccine $PcrV_{28\text{-}294}$-$OprI_{25\text{-}83}$-$Hcp1_{1\text{-}162}$ (POH), containing PcrV, OprI and a vital component of the type VI secretion system Hcp1, was evaluated in murine pneumonia and burn models. It was found that the POH vaccination significantly triggered T-cell responses and proliferation, and protected mice against clinical *P. aeruginosa* strains (Yang et al., 2017). Development of multivalent vaccines may provide a means of protecting against *P. aeruginosa* infections in the future.

6.6. Nanoparticles

Currently, nanoparticles have gained considerable attention for the treatment of a number of diseases, including cancer and bacterial infectious diseases. Nanoparticles are tiny materials having a size of less than 100 nm and a large surface area to mass ratio that have been used in a variety of chemical, biological and biomedical applications (Jeevanandam et al., 2018; Salata, 2004). The nanoparticles used for their antimicrobial activity have high penetrability into bacterial membranes, can disrupt biofilm formation, possess multiple antimicrobial mechanisms, and are good carriers of antibiotics (Wang et al., 2017). Metallic and antimicrobial agent-loaded nanoparticles have been extensively studied for the prevention of *P. aeruginosa* infections (Chatterjee et al., 2016). For instance, silver nanoparticles are effective antimicrobial agents that produce silver ions responsible for inhibition of bacterial enzymatic systems including DNA synthesis (Wang et al., 2017). The silver nanoparticles have shown significant antimicrobial effects on *P. aeruginosa* clinical strains, effectively killing *P. aeruginosa* and inhibiting its growth *in vitro*. Moreover, silver nanoparticles showed low cytotoxicity to mammalian cells, although this requires further testing *in vivo* (Salomoni et al., 2017).

As mentioned earlier, nanoparticles are able to deliver antimicrobial agents such as antibiotics to bacteria (Salata, 2004). Kwon et al. (2017) engineered porous silicon nanoparticles with a novel antimicrobial peptide comprising membrane-interacting peptides fused with a synthetic bacterial toxin. This engineered nanoparticle was found to improve the survival rate and the bacterial clearance in a mouse model of *P. aeruginosa* lung infection. In addition, attachment of antibiotics to nanoparticle surfaces has been found to significantly enhance the efficacy of both antibiotics and nanoparticles. In this regard, silver nanoparticles attached to ampicillin have a higher killing rate of ampicillin-resistant *P. aeruginosa* isolates *in vitro* compared to the silver nanoparticles without ampicillin bound (Brown et al., 2012).

The disadvantage of using nanoparticles is their potential toxicity in humans (Elsaesser and Howard, 2012). Nanoparticles are very reactive due to their high surface area to mass ratio, which may cause unwanted reactions in the human body. Moreover, they are easy to transport to distant organs and can induce systemic toxicity (Gwinn and Vallyathan, 2006; Yildirimer et al., 2011). The use of nanoparticles to treat bacterial pulmonary infection needs to be very carefully considered, because inhalation of nanoparticles has been found to induce pulmonary inflammation and can cause cardiovascular effects (Inoue et al., 2006; Miller et al., 2017). One study reported that carbon nanoparticles could aggravate the LPS-induced lung inflammation in mice (Inoue et al., 2006). Although nanoparticles have shown great antimicrobial activity during *in vitro* and *in vivo* experiments, they are still confined to preclinical stage experiments owing to their potential side effects. Future studies that utilize nanoparticles need to focus on the material selection, size and administered dose of nanoparticles, which may optimize the functioning and reduce the toxicity of nanoparticles in clinical practice.

6.7. Antimicrobial peptides

Antimicrobial peptides (AMPs), also called host defense peptides,

are produced by a variety of organisms, from bacteria to animals, and they are active against a broad range of microorganisms (Toke, 2005). The mode(s) of action of AMPs is not fully understood. It is generally accepted that AMPs target the cytoplasmic membrane, leading to cell death (Park et al., 2011). In addition to antimicrobial activity, AMPs have also been found to possess anti-biofilm and immunomodulatory properties (Chung and Khanum, 2017; Hancock et al., 2016; Pletzer et al., 2016). As a consequence of their broad-spectrum activity, AMPs have been suggested as an alternative to conventional antibiotics to combat bacterial infections; AMPs demonstrate rapid killing kinetics, low levels of induced resistance and low toxicity to host (Gordon et al., 2005; Hancock et al., 2016). Numerous antimicrobial peptides including GL13K, LL-37, T9W, NLF20, cecropin P1, indolicidin, magainin II, nisin, ranalexin, melittin and defensin have displayed potent antimicrobial effects against *P. aeruginosa* through either direct bactericidal effects or disruption of biofilms (Dosler and Karaaslan, 2014; Giacometti et al., 1999; Hirt and Gorr, 2013; Papareddy et al., 2016; Wnorowska et al., 2015; Zhu et al., 2015). Furthermore, some AMPs have shown synergy with conventional antibiotics against many bacteria, including *P. aeruginosa*, by promoting antibiotic uptake, disrupting biofilm formation or inhibiting bacterial quorum sensing (Grassi et al., 2017; Zhou and Peng, 2013). For instance, it has been shown that a combination of GL13K with tobramycin increased the clearance of *P. aeruginosa* biofilm (Hirt and Gorr, 2013). Zheng et al. (2017) observed that the minimum inhibitory concentration of cecropin A2 against *P. aeruginosa* clinical isolates was reduced 8-fold when it was combined with tetracycline *in vitro*. Moreover, this combination increased the survival rate of *P. aeruginosa* PA14-infected *Galleria mellonella* larvae *in vivo*. On the other hand, the disadvantages of AMPs include hemolytic activity in host cells; reduced activity based on salt, serum, and pH sensitivity; rapid degradation in the human body due to susceptibility to proteolysis; and the high cost of AMP production (Aoki and Ueda, 2013; Gordon et al., 2005).

6.8. Electrochemical scaffolds

A recently suggested alternative strategy for eliminating bacterial infections is to utilize electrochemical scaffolds to generate a low but constant concentration of H_2O_2 that is sufficient to destroy bacterial biofilms and allow better antibiotic penetration (Sultana et al., 2015). In this regard, a study by Istanbullu et al. (2012) indicated that the H_2O_2 produced by a steel surface greatly reduced *P. aeruginosa* biofilm formation. A more recent study by Sultana et al. (2016) demonstrated that an electrochemical scaffold was able to enhance tobramycin susceptibility of *P. aeruginosa* PAO1 and effectively eradicate persister cells in biofilms. To date, electrochemical scaffolds have not been implanted into patients, thus the clinical efficacy of this approach remains to be demonstrated.

7. Concluding remarks

The treatment of *P. aeruginosa* infections continues to be a significant challenge. The antibiotic resistance in *P. aeruginosa* is multifactorial in that it can occur through innate, acquired or adaptive mechanisms. The diversity of antibiotic resistance mechanisms contributes to the development of multidrug-resistant strains, and makes conventional antibiotics ineffective for the treatment of *P. aeruginosa* infections. Furthermore, the formation of *P. aeruginosa* biofilms and persister cells are responsible for persistent and recalcitrant infections in CF patients. Over the past few decades, there has been progress regarding the development of new antibiotics with novel modes of action, and resistance to modification by bacterial enzymes along with improvements to drug delivery efficiency. However, *P. aeruginosa* has a remarkable capacity to develop or acquire new resistance mechanisms to these new antibiotics so that the overuse and misuse of antibiotics pose serious concerns for public health. The non-antibiotic therapeutic approaches, especially quorum sensing inhibition, phage therapy and the use of nanoparticles, have shown significant antimicrobial effects against antibiotic-resistant strains of *P. aeruginosa in vitro* or in animal models, and they are being considered as alternatives or adjuncts to conventional antibiotics. However, to date, few of these newer approaches have proceeded to clinical practice due to high cost, side effects and safety concerns. Moreover, *P. aeruginosa* is a highly versatile microbial pathogen that possesses one of the most complex regulatory networks in bacteria. Any interference may potentially cause indirect downstream effects in its cellular physiology and complications of infection in patients. A better understanding of the host-bacteria as an integrated system in response to treatment is necessary for the development of innovative therapeutic strategies to fight against this extremely problematic human pathogen. Development of new antimicrobial agents and alternative strategies for prevention and treatment of *P. aeruginosa* infections is a long and winding road. Future studies need to focus on the improvement of those novel strategies that leads to reduced side effects, and increased safety and effectiveness in clinical trials. Since *P. aeruginosa* utilizes a multifaceted antibiotic resistance strategy, the most effective future treatments will likely require combinational therapies, where novel treatments and traditional treatments, such as conventional antibiotics, are combined to successfully eradicate this pathogen from vulnerable, immunocompromised patients.

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