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Antibiotic resistance in *Pseudomonas aeruginosa* biofilms: Towards the development of novel anti-biofilm therapies

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ABSTRACT

The growth of bacteria as structured aggregates termed biofilms leads to their protection from harsh environmental conditions such as physical and chemical stresses, shearing forces, and limited nutrient availability. Because of this highly adapted ability to survive adverse environmental conditions, bacterial biofilms are recalcitrant to antibiotic therapies and immune clearance. This is particularly problematic in hospital settings where biofilms are a frequent cause of chronic and device-related infections and constitute a significant burden on the health-care system. The major therapeutic strategy against infections is the use of antibiotics, which, due to adaptive resistance, are often insufficient to clear biofilm infections. Thus, novel biofilm-specific therapies are required. Specific features of biofilm development, such as surface adherence, extracellular matrix formation, quorum sensing, and highly regulated biofilm maturation and dispersal are currently being studied as targets to be exploited in the development of novel biofilm-specific treatments. Using *Pseudomonas aeruginosa* for illustrative purposes, this review highlights the antibiotic resistance mechanisms of biofilms, and discusses current research into novel biofilm-specific therapies.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium and an opportunistic pathogen. It is capable of causing both acute and chronic infections and is one of the most common causes of nosocomial infections. In several hospital settings, including medical device-related and lung infections, it causes chronic infections in which the biofilm lifestyle predominates. For example, it is notorious for causing chronic lung infections, which lead to eventually-fatal lung deterioration, in individuals with the genetic disorder cystic fibrosis (CF) (Blanc et al., 1998). Cystic fibrosis (CF) is a genetic disorder due to recessive mutations in the CF transmembrane regulator gene which regulates chloride transport across epithelia. These mutations lead, amongst other phenotypes, to hyperinflammation and a reduced ability to clear bacteria by mucociliary action. As a result, bacteria more readily colonize the lungs. *P. aeruginosa* grows in the CF lung as chronic biofilm infections that cannot be cured, despite the use of a range of potent antibiotics and supportive therapies, and persist for life (Burns et al., 2001; Kolak et al., 2003; Lindsay and von Holy, 2006; Rudkjøbing et al., 2012; van Belkim et al., 2000). The adaptive biofilm mode of growth, often triggered by association with a surface and/or stress, is highly resilient to physical disruption and cell killing by external stresses in the environment. The hardiness of biofilms and their resistance to current antibiotics as well as host immune clearance mechanisms has led to a growing problem in health-care settings. Biofilm infections commonly occur in patient tissues or on body surfaces as well as the abiotic surfaces of medical devices such as joint and organ replacements, catheters (Brouqui et al., 1995), indwelling venous and urinary catheters, stents and ventilators (Camins, 2013; Chenoweth and Saint, 2013). Pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, and *Haemophilus influenzae*, are the leading causes of biofilm infections, and in addition to adaptive antibiotic resistance are demonstrating increasingly diminishing responses to antibiotic treatment (Davies, 2003; Folkesson et al., 2012; Høiby et al., 2010). *P. aeruginosa* regularly develops as biofilms in healthcare-associated infections, and these are difficult to clear with antibiotic therapy alone (Bjarnsholt et al., 2009; Boucher et al., 1997; Worlitzsch et al., 2002). Indeed *P. aeruginosa* has become one of the most important model organisms for the study of biofilms due to the relative ease of growing consistent and reproducible structured biofilms under laboratory

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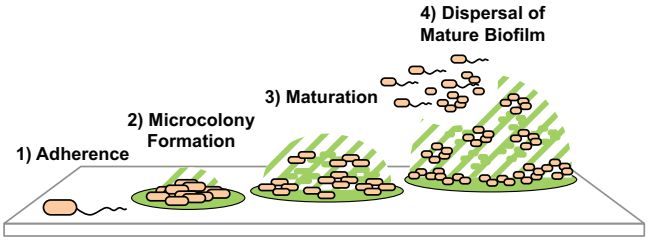

Fig. 1. Biofilm formation progresses through 4 general phases of development. (1) Initial attachment of a cell to a surface is the starting point for the formation of a biofilm. Changes in gene expression lead to the down-regulated expression of polar flagella and up-regulated expression of Type IV pili. Bacteria adhere to the surface using these surface structures and start to produce extracellular matrix, making attachment even stronger. (2) Within 24 h microcolony formation occurs over several rounds of cell division. Continued heightened expression of Type IV pili and secretion of components of the extracellular matrix provides very strong adherence of cells to the surface and strong association with other cells as well as initiating protection from the environment. (3) Over 24–72 h continued growth leads to the formation of a mature biofilm structure. Subpopulations of cells with distinct morphologies develop as gradients in quorum sensing molecules and nutrients exist between the exterior and interior layers of a biofilm microcolony. (4) After more than 48 h, quorum-sensing signaling, external cues and physical disruption lead to some cells from the outer surface of the biofilm colony becoming motile and dispersing.

conditions, as well as its relevance as an opportunistic pathogen in hospital settings. Through discussion of biofilm biology and our understanding of antibiotic resistance in biofilms using *P. aeruginosa* as a model, this review will highlight recent work in developing biofilm-specific therapies for eventual use in the clinic. The major mechanisms of intrinsic, acquired and adaptive resistance in *P. aeruginosa* biofilms will be discussed, stressing the importance of why new strategies are required. In addition, research performed outlining the exploitable characteristics of biofilms and the development of anti-biofilm therapeutics will also be discussed.

2. *Pseudomonas aeruginosa* biofilm development

Bacterial biofilms are structured colonial aggregates of cells encased in an extracellular matrix. Common features of all bacterial biofilms are: (a) triggering upon association with a surface that can be biotic or abiotic, (b) the specific production and secretion of an extracellular matrix, that can include exopolysaccharides, DNA and/or proteins (Barken et al., 2008; Friedman and Kolter, 2004; Klausen et al., 2003), (c) the coordinate maturation of biofilms often involving cell-to-cell communication through quorum sensing, (d) observable developmental stages and the growth of subpopulations of cells within a biofilm colony (Davies et al., 1998; De Kievit et al., 2001), and (d) dispersal of planktonic cells from the biofilms to enable establishment of new biofilm colonies at distant sites (de la Fuente-Núñez et al., 2013). Biofilms display a much higher resistance to killing by most antimicrobial compounds, by about 10–1000-fold, when compared to free swimming, planktonic cultures (Hoyle and Costerton, 1991). The high level of resistance observed in biofilms is proposed to result from multiple factors associated with adaptive changes in gene expression accompanying the biofilm growth state and/or external stresses, and the inherent properties of biofilm structures.

As shown in Fig. 1, the first step in biofilm formation begins with the irreversible adherence of cells to a surface (e.g. on an abiotic – plastic, metal, glass, environmental – or biotic surface). This involves the action of pili and flagella. Type IV pili are filamentous protein complexes that represent a major mechanism of adherence in *P. aeruginosa* and are of vital importance for the initial attachment of cells, as well as proper maturation of biofilms (O'Toole and Kolter, 1998; Toutain et al., 2007). Motile bacteria, like *Pseudomonas*, possess flagella that enable free-swimming (planktonic) bacteria to approach a surface but also play a vital role in adherence to the surface and initiation of the transition from free-swimming bacteria to sessile, adherent bacteria (Caiazza et al., 2007; O'Toole and Kolter, 1998; Toutain et al., 2007).

Through cell growth and division, aggregate cells develop into a microcolony (Sriramulu et al., 2005) and adherence becomes stronger due to the initiation of matrix production that aids in strong surface adherence. In nature, independent microcolonies can merge together to form a mat that can grow to a size that is visible to the naked eye. In medicine, biofilms can cover the entire surface of wounds such that they have to be removed surgically. Biofilm colonies are characterized by closely packed cells contained within an extracellular polymeric substance (EPS) matrix that allows nutrients and small molecules to readily permeate through the biofilm mass. Interestingly, cells in the interior of biofilm microcolonies grow and divide much more slowly than the outer layers of cells (Werner et al., 2004), since the outermost bacteria filter out and utilize nutrients faster than diffusion can replenish the inner layers of the biofilm.

The EPS matrix is in part secreted by cells within a biofilm and develops in the early stages of growth. Exopolysaccharides, extracellular DNA (eDNA), proteins, lipids, and biosurfactants can make up the EPS matrix which promotes the strong adherence of cells to the surface and provides structure to a biofilm (Barken et al., 2008; Friedman and Kolter, 2004; Klausen et al., 2003). In *P. aeruginosa*, the Pel and Psl polysaccharides are major contributors to biofilm maturation and structure (Jackson et al., 2004; Ma et al., 2009; Vasseur et al., 2005), although alginate has also been proposed to have a role. In addition, eDNA appears to play an important role in maintaining the interconnection of cells in the early developmental stages of biofilms as well as being involved to some extent in antibiotic resistance (discussed below) (Allesen-Holm et al., 2006; Whitchurch et al., 2002).

Biofilm maturation is a very sophisticated process involving changes in the expression of dozens to hundreds of genes (Whiteley et al., 2001), triggered by the biofilm development program. This program is highly regulated.

A critical global regulatory circuit is the stringent response. The stringent response is mediated by the nucleotide alarmones guanosine tetra- and penta-phosphate (collectively (p)ppGpp), and is triggered by cellular stress (including amino acid, carbon source, nitrogen, phosphorus, iron or lipid starvation as well as when heat and oxidative stresses) (Potrykus and Cashel, 2008). This leads to induction of (p)ppGpp synthesis by the enzymes RelA and SpoT which then modulates the transcriptional capacity of RNA polymerase affecting biofilm formation (de la Fuente-Núñez et al., 2014), as well as numerous stress and starvation responses. Dozens of other genes and regulatory circuits are involved in biofilm formation (e.g. Table 1), many of which are independent of the global regulatory circuits (stringent response and quorum sensing).

An additional small molecule that is now understood to be important for regulating biofilm development is another unusual nucleotide signalling molecule, 3′,5′-cyclic diguanylic acid (c-di-GMP). Diguanylate cyclases are responsible for synthesizing c-di-GMP and have been found to be encoded in most bacterial genomes and are quite well conserved (Ryjenkov et al., 2005). Indeed, c-di-GMP has been shown to affect functions related to the switching between the motile, single cell state and the surface-associated multicellular biofilm state (Jenal and Malone, 2006). It has been found c-di-GMP is a major regulator of key biofilm features such as the production of the EPS matrix as well as the transition of cells from being motile to becoming sessile, adherent cells of a biofilm (Bomchil et al., 2003; D'Argenio et al., 2002) It is worth stating, in addition, that there are many other such

Table 1
Genes influencing biofilm formation and antibiotic susceptibility in *Pseudomonas aeruginosa*.

PA number	Gene name	Mutant phenotype			References[b]
		Biofilms	Resistances[a]	Other	
PA0084	*tssC1*	Normal	TOB^S	Type 6 secretion system deficiency	1
PA0355	*pfpI*	Decreased	CIP^R	Swarming deficient	2
PA0401	–	Decreased	PX^S		3,4
PA0402	*pyrB*	Decreased	COL^R, $LL37^R$, PX^R		4,5
PA0756/57	–	Normal	GEN^S, TOB^S		5
PA0779	*asrA*	Increased	TOB^S	Reduced heat shock response	4,6
PA1163	*ndvB*	Normal	GEN^S, TOB^S	Reduced expression of ethanol oxidation pathways	7,8
PA1180	*phoQ*	Decreased	AMK^R, KAN^R, PX^R	LPS modification deficiencies	9,10
PA1588	*sucC*	Decreased	PX^S		2,4,11,12
PA1799	*parR*	Increased	COL^S, PX^S		4,11,12
PA1801	*clpP*	Decreased	CIP^S	Swarming deficient	3
PA1803	*lon*	Decreased	CIP^S	Reduced cytotoxicity, swarming deficient	9,13
PA1875–PA1877	–	Normal	CIP^S, GEN^S, TOB^S		14
PA2070	–	Normal	GEN^S		5
PA2523	*czcR*	Decreased	IPM^R	Reduced uptake of Zn^{2+},Cd^{2+}, Co^{2+}, Cu^{2+}; Catabolite repression	15,16
PA2621	*clpS*	Decreased	ATM^R, CAZ^R, PIP^R		2
PA3006	*psrA*	Decreased	IND^S, PX^S	Reduced swarming	17
PA3920	–	Decreased	TOB^R		4,19
PA4459	*lptC*	Decreased	COL^S, IND^S, $LL37^S$, PX^S		4,11,12
PA4725	*cbrA*	Increased	CIP^R, PX^R, TOB^R	Deficient in catabolite repression, swarming, cytotoxicity, etc.	9,13,19
PA4867	*ureB*	Decreased	TOB^S		4,19
PA5033	–	Normal	GEN^S		5
PA5038	*aroB*	Decreased	PX^S		4,11,12

[a] ^{R} = resistant; ^{S} = supersusceptible; *Antibiotic abbreviations*: AMK – amikacin; ATM – aztreonam; CAZ – ceftazidime; CIP – ciprofloxacin; COL – colistin; GEN – gentamicin IND – bovine indolicidin; IPM – imipenem; KAN – kanamycin; LL-37 – human host defence peptide; PIP – piperacillin; PX – polymyxin B; TOB – tobramycin.
[b] Reference code: 1. Zhang et al., 2011; 2. Fernández et al., 2012b; 3. Fernández et al., 2012c; 4. Musken et al., 2010; 5. Zhang et al., 2013; 6. Kindrachuk et al., 2011; 7. Beaudoin et al., 2012; 8. Yeung et al., 2009; 10. Macfarlane et al., 2000; 11. Dötsch et al., 2012; 12. Fernández et al., 2012a; 13. Breidenstein et al., 2012b; 14. Zhang and Mah, 2008; 15. Dieppois et al., 2012; 16. Trias and Nikaido, 1990; 17. Gooderham et al., 2008; 18. Whiteley et al., 2001; 19. Yeung et al., 2011.

regulators; e.g. Yeung et al. (2009) identified 18 such "switch" regulators suggesting that there are multiple systems that regulate biofilm development.

A cell-density-dependent intercellular type of communication called quorum sensing represents another regulatory system. The role of quorum sensing in biofilm maturation is still not fully understood since quorum sensing deficient strains of *P. aeruginosa* are capable of forming biofilm structures, albeit with distinct morphologies from quorum sensing competent parental strains (Davies et al., 1998; De Kievit et al., 2001). The chemical structures of quorum sensing molecules vary greatly across different species of bacteria. One major class in Gram negative bacteria, like *P. aeruginosa*, are the acyl homoserine lactones (AHLs) that are small molecules utilized for quorum sensing signaling during biofilm development. In *P. aeruginosa*, AHL synthesis and response is regulated by the *las* and *rhl* systems (Pesci et al., 1997). An additional quorum sensing system independent of the *las* and *rhl* systems in *P. aeruginosa* involves an orphan autoinducer, the *Pseudomonas* quinolone signal (PQS) (Diggle et al., 2003).

3. Antibiotic resistance mechanisms of *Pseudomonas aeruginosa*

Antibiotics used to treat *P. aeruginosa* infections include a number of β-lactams such as cephalosporins (e.g. ceftazidime), carbapenems (e.g. imipenem, meropenem) and monobactams (e.g. aztreonam). Piperacillin is a penicillin class β-lactam that remains effective in treating most *P. aeruginosa* clinical infections. Other major classes of antibiotics used to treat *P. aeruginosa* infections include aminoglycosides (e.g. gentamicin, tobramycin, amikacin), quinolones (e.g. ciprofloxacin), and, more recently, polymyxins (e.g. colistin, polymyxin B). Separate multi-drug resistant strains of *P. aeruginosa* isolated from clinical environments display a high degree of variability in acquired mutations that lead to resistance suggesting that resistance can develop readily and rapidly (Jeukens et al., 2014). Here the major mechanisms of antibiotic resistance in *P. aeruginosa* that fall under the classifications of intrinsic, acquired and adaptive resistance will be discussed.

3.1. Intrinsic resistance

Intrinsic resistance represents the underlying mechanisms that limit susceptibility to antibiotics in wild type organisms. In Gram-negative bacteria this depends on the semi-permeable outer membrane that limits the rate of uptake of antibiotics. The outer membrane is an asymmetric membrane composed of an inner leaflet of phospholipids and an outer layer of polyanionic lipopolysaccharide (LPS). The permeability of the outer membrane of *P. aeruginosa* is 12–100-fold lower than that of other Gram-negative bacteria, such as *E. coli* (Hancock and Bell, 1988). This low permeability of the *P. aeruginosa* outer membrane serves as a significant barrier to the penetration of antibiotics and therefore, small hydrophilic antibiotics, such as β-lactams and quinolones, pass through the water-filled porin channels relatively slowly, making *Pseudomonas* generally more intrinsically resistant to most antibiotics. Nevertheless, although low outer membrane permeability

plays a significant role in restricting antibiotic uptake, it is not a stand-alone resistance mechanism since despite slower uptake, eventually antibiotics will equilibrate across the membrane.

The slow uptake of antibiotics synergizes with other mechanism of intrinsic resistance including, e.g. efflux pumps and periplasmic β-lactamases, leading to a high level of resistance to many clinically relevant antibiotics in *P. aeruginosa*. Multi-drug efflux systems (e.g. the constitutive MexAB-OprM and MexXY-OprM systems of *P. aeruginosa*) and inactivating enzymes (e.g. the low level but inducible chromosomal β-lactamase AmpC of *P. aeruginosa*), take advantage of the reduced flow of antibiotics across the outer membrane to actively pump and/or degrade antibiotics as they pass through.

Recent screens of comprehensive libraries of transposon-tagged insertion mutants for increased antibiotic susceptibility compared to the wild type strain (i.e. intrinsic resistance genes) have led to the identification of novel candidate mechanisms involving more than a hundred genes that belong to different functional families in the intrinsic resistome of *P. aeruginosa* (Alvarez-Ortega et al., 2010; Breidenstein et al., 2008), and exactly how many of these work remains to be elucidated. For example, Breidenstein et al. (2008) identified 35 genes involved in intrinsic resistance to ciprofloxacin.

3.2. Acquired resistance

While intrinsic resistance is present in the cell without any prior exposure to antibiotics, acquired resistance arises via antibiotic exposure, resulting in selection of a large array of chromosomal gene mutations (Alvarez-Ortega et al., 2010; Breidenstein et al, 2008; Schurek et al. 2009), or acquisition of resistance genes through genetic elements such as plasmids, transposons, interposons and integrons. This is a vast topic and is just outlined here and reviewed elsewhere (Breidenstein et al., 2011). Although acquisition of resistance genes is not as common in *P. aeruginosa* as many other bacteria, it has recently been shown to acquire plasmid-encoded carbapenemases (unique metallo-β-lactamases) that can hydrolyze most β-lactams, including the carbapenems, but not aztreonam (Umadevi et al., 2011). One common mechanism of acquired resistance to the carbapenem β-lactams in *P. aeruginosa* involves mutations leading to loss or reduced production of the OprD specific outer membrane porin channel leading to resistance to the carbapenem imipenem, often increasing the MIC of imipenem by 8–16-fold (Livermore, 2001). Mutations leading to the loss of OprD can be due either to mutation in the *oprD* gene itself or in one of its regulatory proteins (e.g. MexT) (Livermore, 2001). Interestingly, mutations in *mexT* not only reduce the expression of OprD, it concurrently increases the expression of MexEF-OprN resulting in dual resistance to imipenem and to antibiotics that are substrates for this pump (Kohler et al., 1999). Two of the major intrinsic resistance mechanisms of *P. aeruginosa* can also be enhanced by mutations leading to enhanced expression, i.e. *ampD* mutations leading to derepressed chromosomal AmpC β-lactamase and *nalB* mutations leading to de-repression of the MexAB-OprM multidrug efflux pump. For example, overexpression of the AmpC enzyme increases the resistance of *P. aeruginosa* to many β-lactams, including penicillins, cephalosporins, and to a lesser extent the carbapenem meropenem (Langaee et al., 2000). In *P. aeruginosa* clinical isolates, overexpression of AmpC has been predominately associated with mutations in *ampD*, a gene encoding the *ampC* repressor (Langaee et al., 2000), although other mechanisms exist (Alvarez-Ortega et al., 2010). In addition to the chromosomally expressed AmpC β-lactamase, *P. aeruginosa* can also acquire plasmid-encoded β-lactamases. Recently described β-lactamases acquired by *P. aeruginosa* include an extended spectrum β-lactamase (ESBL) that hydrolyzes a wide range of β-lactams, including the broad spectrum cephalosporins and monobactams (Umadevi et al., 2011).

3.3. Adaptive resistance

Adaptive resistance is becoming increasingly recognized as a clinically important mechanism of resistance (Fernández et al., 2011). In contrast to intrinsic and acquired resistance that are hard wired, adaptive resistance is dependent on the growth circumstances that trigger regulatory events in the cell, and susceptibility usually reverts when the inducing conditions are removed. For example, Greenwood (1975) was one of the first to show that microorganisms exposed to sub-inhibitory concentration of antibiotics could become transiently more resistant to subsequent exposure to that antimicrobial agent, and in some cases, to other agents of the same or different classes.

Adaptive resistance is rapidly induced by specific growth states or conditions, environmental stimuli, or chemical and physical stresses (Fernández et al., 2011). Environmental cues that have been reported to induce this type of resistance include antibiotics and biocides, pH, heat shock, DNA stress (SOS response), anaerobiosis, polyamines, cations, and nutrient deficiencies, as well as adaptive social behaviors such as biofilm formation and swarming motility (Fernández et al., 2011). Adaptive resistance appears to involve many genes including those found during extensive characterizations of the resistomes (i.e. all genes that when mutated lead to altered susceptibility) for different antibiotics (e.g. Alvarez-Ortega et al., 2010; Breidenstein et al., 2008, 2012a; Schurek et al., 2009). For example, it is known that sub-inhibitory concentrations of ciprofloxacin cause dysregulation of more than 900 genes (Brazas and Hancock, 2005), including a number that match to the ciprofloxacin resistome (Breidenstein et al., 2008). Recent studies have emphasized the role of complex regulators, including ATP-dependent proteases in heat shock-mediated adaptive aminoglycoside resistance and DNA-stress mediated adaptive fluoroquinolone resistance (Breidenstein et al., 2012a; Kindrachuk et al., 2011; Table 1), and many others, including two-component regulators *phoQ, czcR*, *psrA*, and *cbrA*, that are able to impact on resistance to multiple antibiotics, biofilm formation and swarming motility (e.g. Table 1). Adaptive resistance is likely to contribute to discrepancies that are often observed between in vitro susceptibility assessments for *P. aeruginosa* isolates and treatment outcomes in infected patients.

In *P. aeruginosa*, one of the best characterized mechanisms of adaptive resistance is the development of resistance to polymyxins and cationic antimicrobial peptides in response to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} cations (Macfarlane et al., 1999; McPhee et al., 2003) or antimicrobial peptides themselves (Fernández et al., 2012a,b,c; McPhee et al., 2003). Adaptation to limiting divalent cations is controlled by PhoPQ and PmrAB (Macfarlane et al., 1999; McPhee et al., 2003). Alterations in the permeability (self-promoted uptake pathway) of the outer membrane can be induced, through the PhoPQ and PmrAB pathways, by a divalent cation deficiency due to the high concentrations of extracellular DNA in biofilms (Mulcahy et al., 2008), resulting in tolerance to polymyxins and other cationic antimicrobial peptides. As described above this triggers the addition of aminoarabinose to the 1′ and 4′ phosphates of lipid A of LPS, reducing the net negative charge of LPS and limiting the uptake of polycationic antimicrobials across the outer membrane. Moreover, recent studies have shown that sub-inhibitory concentrations of polymyxins and cationic antimicrobial peptide are likely the primary in vivo mechanism for induction of adaptive resistance via LPS modification due to dysregulation of the *arnBCADTEF* operon that arabinosaminylates LPS thus decreasing self-promoted uptake of the inducing polycations (McPhee et al., 2003). However, neither PhoPQ nor PmrAB mediate

this effect and instead different peptides work through either ParRS or CprRS or both to mediated adaptive resistance (Fernández et al., 2012a,b,c).

4. Antibiotic resistance in *Pseudomonas aeruginosa* biofilms

Antibiotic resistance in biofilms is complex and results from contributions of intrinsic, acquired and adaptive mechanisms. Most notably, biofilm specific features such as the differential expression of multiple gene networks, extracellular matrix, and the metabolic heterogeneity of sub-populations within a biofilm colony are major contributors to antibiotic resistance. It was previously suggested that the biofilm structure led to reduced antibiotic uptake. More recently it has been suggested that antibiotics are able to readily diffuse through microcolonies (Anderl et al., 2000; Walters et al., 2003). Consistent with this, the reduced activity observed for positively charged classes of antibiotics like polymyxins and aminoglycosides is at least in part due to the presence of anionic eDNA rather than an inability to diffuse across the biofilm mass (Kumon et al., 1994). Intrinsic mechanisms of resistance are present in biofilms but largely work in synergy with acquired and adaptive mechanisms that contribute to antibiotic resistance in biofilms.

Acquired resistance occurs commonly in biofilms largely due to horizontal gene transfer between strains and species (Molin and Tolker-Nielsen, 2003; Thomas and Nielsen, 2005). A complete description of these mechanisms is beyond the scope of this review, and we refer the reader to a recent review (Breidenstein et al., 2011). However critically it is thought that resistance enzymes like β-lactamase might be more concentrated within a highly structured biofilm, exacerbating their influence on resistance (Bagge et al., 2004).

The importance of adaptive resistance is becoming increasingly appreciated for its major contribution to antibiotic resistance in biofilms (Fernández et al., 2011). As mentioned above, *P. aeruginosa* cells in a biofilm state of growth are significantly more resistant to antimicrobial agents than in the planktonic state (Hoyle and Costerton, 1991; de la Fuente-Núñez et al., 2013). This has been ascribed to a range of different factors including a complex array of adaptive gene expression changes, some of which influence antibiotic susceptibility, the low metabolic state of organisms deep within the biofilm, and the higher concentration of extracellular antibiotic-degrading enzymes trapped within the biofilm matrix. Of these, the most intriguing involves adaptive changes in gene expression that accompany the switch to the biofilm mode of growth. The phenomenon of adaptive resistance, although known for decades, was poorly recognized in the clinic for many years since conventional susceptibility tests were unable to detect this type of resistance which reverts when cells are grown under lab conditions.

Although adaptive resistance provides tolerance to antimicrobial agents which is dependent on environmental conditions or the growth state, it is likely that it also contributes to or enables the stepwise accumulation of mutations in biofilms, leading to more conventional acquired resistance. The increasing accumulation of acquired mutations giving rise to antimicrobial resistance has been observed in the laboratory (Amini et al., 2011; Macià et al., 2011) and during the development of heterogeneous populations of *P. aeruginosa* in chronic lung infections (Wang et al., 2010; Xu et al., 1998), with the latter phenomenon being exacerbated by the development of mutations in mutator genes (Macià et al., 2011).

As mentioned above, many antimicrobial agents penetrate reasonably well into biofilms, although certain nutrients such as oxygen might be limiting in the interior part of biofilms and thus create an anaerobic or microaerophilic environment (Borriello et al., 2004; Walters et al., 2003; Xu et al., 1998). This anaerobic environment within biofilms would likely impact directly on aminoglycoside antibiotic activity (Kindrachuk et al., 2011) due to decreased energy dependent uptake (Hancock, 1981), as well as by triggering changes in gene expression. These and other changes brought about by the dense aggregation of bacteria within biofilms, and consequent limiting nutrient availability, suggests that bacteria deep within biofilms have reduced metabolic activity and lower rates of cell division than those closer to the surface of biofilms. Such cells in biofilms that survive antibiotic treatment due to non-mutational mechanisms have been termed “persister” cells. Persisters have been found to develop more effectively when biofilms are exposed to antimicrobial treatment, and they are considered a significant contributor to the resilience of biofilm infections (Balaban et al., 2004; Keren et al., 2004; Mulcahy et al., 2010; Shah et al., 2006). The phenomenon of persistence in biofilms is still not fully understood, however, recent evidence suggests that there is an underlying genetic contribution to the development of persistent cells after antibiotic treatment (Balaban et al., 2004; Mulcahy et al., 2010). Nevertheless persistence in biofilms is likely due to cells that have low levels of transcription, translation, and genome replication, and/or low metabolic activity. Since these encompass many targets for clinically relevant antibiotics, which also tend to work better on metabolically-active cells, they would limit susceptibility of persisters. However it is quite possible that persistence is also an adaptive state. For example, it was recently suggested that the stringent response, which is highly influential in biofilm development (de la Fuente-Núñez et al., 2014), also contributes to the development of persisters (Maisonneuve et al., 2013). Once the antibiotic is removed from the surrounding environment, these antibiotic resistant persisters can drive the re-establishment of the biofilm.

While the molecular mechanisms underlying adaptive resistance are just starting to be understood due to their complexity, continuing research is required to gain a more complete understanding of this phenomenon and aid in efforts to design more effective treatment programs. This is especially true in cases where the levels of the antibiotic in the patient might fall into the sub-inhibitory range (a significant risk when chronic biofilm infections are present) and/or the local conditions or the in vivo growth state trigger resistance to certain antibiotics.

5. Combination therapies, novel modifications and re-visiting old antibiotics

Combinations of antibiotics are already widely used in the clinic since they help to prolong the effective life of each of the drugs in the combination, and delay the development of wide-spread antibiotic resistance. These combinations, often involving structurally distinct classes of antibiotics, are usually selected because they demonstrate synergy. However synergy is rarely tested with organisms in the biofilm state which has limited progress. Indeed, extensive work is required to define the optimized combinations and/or to develop compounds that work optimally in combination with existing antibiotics (e.g. Reffuveille, 2014).

Nevertheless, combinations do provide the potential to overcome the above-described high adaptive resistance of biofilms to many antibiotics. For example, antibiotic combinations have shown reasonable success in clearing *P. aeruginosa* pulmonary biofilm infections (Herrmann et al., 2010; Tre-Hardy et al., 2010; Yu et al., 2012). In addition, the polymyxins are being utilized as a last hope drug for *P. aeruginosa* infections (Falagas and Bliziotis, 2007) and combinations with tobramycin have proven to be highly effective against biofilm infections in patient trials (Herrmann et al., 2010). Another combination that has shown promise involves

the aminoglycoside tobramycin and macrolide clarithromycin. Each have only moderate activity against biofilms cf. free swimming planktonic cells, but when used in combination demonstrate synergy and are significantly more effective at clearing biofilms (Tre-Hardy et al., 2010). However, the mechanistic basis for these synergistic effects is still poorly understood. Similar combinations of tobramycin with another macrolide, azithromycin, actually led to a reduced effectiveness in vitro compared to either antibiotic was used individually (Tre-Hardy et al., 2010), while the use of tobramycin with the β-lactam aztreonam provided no additional efficacy over tobramycin alone in such cases (Yu et al., 2012).

While certain combinations of different classes of antibiotics have synergistic effects by acting jointly on cells, other combinations of antibiotics are effective in clearing biofilms by targeting separate sub-populations (Herrmann et al., 2010). For example, ciprofloxacin is more effective at killing cells from the outer layers of a biofilm colony, while colistin, shows the opposite trend preferentially killing cells in the interior of biofilms (Haagensen et al., 2007; Pamp et al., 2008). The combination of colistin and ciprofloxacin is thus far superior to monotherapy (Herrmann et al., 2010).

Efforts are also being pursued to create more effective conventional treatments against biofilm infections by modifying existing antibiotics. For example, new cephalosporins have proven to be effective against multi-drug resistant strains of *P. aeruginosa* in biofilm infections and in respiratory infections of *P. aeruginosa* (Juan et al., 2010; Moya et al., 2010; Riera et al., 2010). However, the greatest difficulty in treating biofilms is their resilient nature and multi-drug resistance is one contributor. This clearly underscores that novel treatments, tailored for treating biofilm infections, are needed to address the unique growth state of biofilms, and it is telling that not a single antimicrobial is available clinically that has been developed for this purpose despite the massive importance of biofilms in the clinic.

6. Novel strategies for treating biofilm infections

The high resistance of biofilms to antimicrobial treatment requires the development of biofilm-specific therapeutics, and many are being pursued (de la Fuente-Núñez et al., 2013). While biofilms are highly adapted to survive perturbations and environmental stresses, there are specific aspects of the biofilm growth state that may be exploited for treating biofilm infections. Inhibiting adherence to surfaces, and quorum sensing and stringent response signaling for maturation, and modulation of dispersal are among the most promising targets.

6.1. Disruption of cellular adherence to surfaces

P. aeruginosa biofilms can be found on medical surfaces such surgical equipment and indwelling medical devices including artificial joints and catheters. By binding to these surfaces, *P. aeruginosa* can grow as biofilms as well as initiate chemical modification of surfaces to enhance subsequent adherence of *Pseudomonas* and other bacteria during subsequent colonizations (Davis et al., 2011; Davis et al., 2013). Multiple approaches have been taken to preventing such colonization by coating medical surfaces for example with silver particles, which have demonstrated some success at limiting the formation of surface biofilms (Li et al., 2010; Mijnendonckx et al., 2013). However, these methods are not an appropriate substitute for already established sterilization and decontamination procedures. Other work has actively pursued novel compounds to inhibit the adherence of uropathogenic *Escherichia coli* (UPEC) to surfaces, utilizing pilicides and curlicides which inhibit the adhesins used to attach to surfaces (Chorell et al., 2012; Piatek et al., 2013; Pinkner et al., 2006).

Despite decades of research into the development of strategies for treating infections and prevent growth on indwelling abiotic surfaces, the major focus for treating device related biofilms in hospital settings has been the removal and replacement of the device. This also occurs in chronic infections such as abscesses and wounds, where surgical removal of infected tissue, and consequent removal of biofilms followed by local or systemic antibiotic treatment.

6.2. Enhancing dispersal of cells in biofilms

Increasing the dispersal of biofilm cells is an attractive strategy being explored as a biofilm-specific therapy. This approach seeks to overcome adaptive and intrinsic resistance mechanisms that are characteristic of biofilm cells. It should be stated that the release of large numbers of planktonic cells through such a strategy carries its own dangers and dispersal agents will likely be used in combination with conventional antibiotics to kill the more susceptible planktonic cells. One of the earliest examples of biofilm-specific therapy that focused on dispersal of biofilms was the use of Nitric Oxide (NO), which acts as a signal to trigger biofilm dispersal (Barraud et al., 2009a,b). Mechanistically, it was suggested that signaling through NO triggers quorum sensing signaling and reduces c-di-GMP synthesis promoting the dispersal of cells from the mature biofilm.

Other methods of disrupting the structure of biofilms have focused on directly digesting the extracellular matrix of biofilms. For instance, direct treatment of biofilms with deoxyribonuclease (DNase) leads to enzymatic digestion of the eDNA which is an important structural component of the extracellular matrix of biofilms (Allesen-Holm et al., 2006). The application of DNase treatment concomitantly with antibiotics has been shown to successfully clear biofilm infections (Shakir et al., 2012).

6.3. Quorum sensing inhibitors

An active area of research, referred to as quorum quenching, involves the use of small molecules that inhibit proper signaling by quorum sensing pathways. The goal of using of a quorum sensing inhibitor as a therapeutic in treating biofilm infections is to prevent the full maturation of biofilms and deny bacterial cells the full protection from traditional antimicrobial agents that is provided by a mature biofilm (Rampioni et al., 2014; Scutera et al., 2014). Multiple approaches have been taken towards disrupting quorum sensing including inhibiting the synthesis of quorum sensing molecules, reducing the availability of the secreted quorum sensing molecules, or blocking the reception of a molecule. The most promising approach to quorum quenching involves targeting acyl-homoserine lactones, which represent the major quorum sensing molecules of *P. aeruginosa*. The use of quorum sensing inhibitors to block acyl-homoserine lactone signaling has shown some modest success in limiting biofilm growth under laboratory conditions (Jakobsen et al., 2012; Scutera et al., 2014). However, a major short-coming of using quorum sensing inhibitors as a novel therapeutic in the clinic is the moderate role of quorum sensing in biofilm infections.

6.4. Anti-biofilm peptides

A recent area of research has been aimed at developing novel therapeutics based on cationic amphipathic peptides found in multiple forms of life (Hancock and Sahl, 2006; Jenssen et al., 2006). Small synthetic peptides engineered from naturally derived antimicrobial peptides have been both rationally and randomly designed for optimal effectiveness against microbes (Fjell et al.,

2012), extrapolating from the design principles of natural peptides, which are 12–50 residues in length with 2–9 positively charged and ~50% hydrophobic amino acids. An exciting new prospect is provided by a recent finding that some of these synthetic peptides are able to preferentially and potently kill cells in biofilms. The breakthrough observation was that the naturally produced human host defense peptide LL-37, at one-eighth of its minimal inhibitory concentration (MIC) inhibited *P. aeruginosa* biofilm formation and dissolved pre-existing biofilms (Overhage et al., 2008). Intriguingly, the bacterial-derived cationic lipopeptides polymyxin/colistin has no preferential activity vs. biofilms.

Based on this observation, small peptides were screened for and demonstrated to have excellent broad spectrum anti-biofilm activity (de la Fuente-Núñez et al., 2012). Interestingly, while they share many similarities in their chemical characteristics, the peptide sequence requirements that govern anti-biofilm activity are different from those that contribute to direct antimicrobial activity against planktonic cells (de la Fuente-Núñez et al., 2012). For example, peptides with good anti-biofilm activity but weak antibiotic activity were identified, while anti-biofilm peptides demonstrated activity against biofilms formed by *Burkholderia* sp. which in their planktonic state are completely resistant to the activity of known antimicrobial peptides (and most conventional antibiotics; Moore et al., 1986). Preliminary structure activity relationship studies enabled the identification of peptide 1018 with broad spectrum anti-biofilm activity that includes many multi-drug resistant Gram-negative and Gram-positive bacteria (de la Fuente-Núñez et al., 2014). It was demonstrated that peptide 1018 acts by inhibiting the stringent response required for biofilm formation in *Pseudomonas* and other species, by entering cells and marking (p)ppGpp for degradation (de la Fuente-Núñez et al., 2014). This discovery is particularly exciting since peptide 1018 blocks biofilm growth, causes killing of cells in the mature biofilm state and enhances dispersal (de la Fuente-Núñez et al., 2014), while demonstrating excellent synergy with conventional antibiotics (Reffuveille, 2014). In addition, it has very potent anti-infective immune modulating activity in animal models (e.g. Achtman et al., 2012; Rivas-Santiago et al., 2013).

6.5. Bacteriophage (phage) therapy

Phages are bacterial viruses that do not infect eukaryotic cells. Lytic phages are of substantial interest as novel therapeutics due to their life cycle of replication within a bacterial cell that ultimately results in cell lysis leading to spread of the lytic phage. The use of phages to kill infecting bacteria is being pursued as an alternative to antibiotics. It also is considered interesting for use in biofilm infections based on the close proximity of bacterial cells within a biofilm which enables high local viral titres and rapid spread of phage infections throughout a biofilm colony (Ahiwale et al., 2011; Ryan et al., 2011; Sulakvelidze et al., 2001). In addition, it has been observed that anti-biofilm phage therapy leads to a reduction in the integrity of the extracellular matrix (Glonti et al., 2010). Phages have a high level of species specificity which provides the potential advantage of targeting only specific pathogenic bacteria and leaving the normal flora unaffected. However, one issue with phages as therapeutics is the potential development of phage resistance, counteracted in part by using consortia of phages to delay resistance development and overcome existing strains resistant to specific phages. Other issues concern the influence of phage therapy on the immune system, and the potential for lytic phages to carry genetic material (e.g. encoding resistance genes), which might compromise conventional therapy (Donlan, 2009; Ly-Chatain, 2014; Örmälä and Jalasvuori, 2013). Currently, the use of phages in the clinic has not received regulatory approval and is limited to specific centers. Thus, continued work is required to understand the complex interactions and selective pressures that occur between phages and their host bacteria.

7. Concluding remarks and future directions

Billions of years of selective pressures have given rise to the biofilm mode of growth as a preferred growth state of bacteria, and consequent adaptive antibiotic resistance will always be an obstacle when treating biofilm infections. Nevertheless, the massive complexity inherent in the biofilm development program, and the regulatory switches and environmental cues triggering this program, has meant our understanding of this state is still fragmentary. This will encourage increasingly systems oriented research in many different pathogens.

The development of refined surfaces that hinder the attachment of cells, and the use of novel biocides and anti-biofilm agents, enhancing dispersal and disrupting the natural developmental cycle of biofilms provide good avenues for the development of novel therapies to specifically minimize biofilm growth in hospital environments and treat biofilm infections. Nevertheless, the complexity of the biofilm lifestyle, and the need to consider and maintain the current weapons, i.e. antibiotics, that we have available against infection, means that such approaches are likely to always be adjunctive (i.e. used together with antibiotics). Antibiotic therapies will always be a powerful tool to treat bacterial infections, and it cannot be stressed enough that research into novel antibiotics will always be needed. However, due to the difficulties in treating biofilm infections, biofilm-specific therapies and novel therapeutics that enhance the use of conventional antibiotics are also sorely needed. By actively studying the complex lifestyles of biofilms we should be able to identify new targets and eventually minimize the suffering of individuals with biofilm infections.

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