Peering into the matrix: A look at biofilms and their inherent antibiotic resistance

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Abstract

Biofilms are increasingly being regarded as the predominant form of bacterial growth in natural settings. These structures consist of bacterial cells immobilized at a surface and encased in a self-produced matrix of extracellular polymeric substances. In clinical settings, biofilms are the cause of persistent infections that are difficult to clear through the action of the host immune system. Biofilm-encased cells are also associated with increased levels of antibiotic resistance compared to their planktonic counterparts. The result is increased morbidity and mortality when biofilms are associated with disease. In this review, the focus of discussion will be the various mechanisms of antibiotic resistance common to biofilms and the role these mechanisms play in the pathogenesis of major clinically-relevant microorganisms. Antibiotic penetration, altered microenvironments, phenotypic variation, and adaptive resistance mechanisms are all key players in the development of antibiotic resistance in bacterial biofilms. Though the relative significance of each individual mechanism varies, when combined they confer extensive protection to the biofilm's cellular populations.

Keywords: biofilms; antibiotic resistance (mechanisms of); clinical applications; disease; antibiotic penetration; altered microenvironments; phenotypic variation; adaptive resistance; review

Biofilms: Prevalence and Formation

Biofilms are highly organized consortia of microorganisms, fixed at a surface and encased in a matrix of extracellular polymeric substances (EPS). EPS generally consist of polysaccharides, proteins, and nucleic acids, secreted by the bacteria in the developing biofilm. These secreted substances, often referred to as the matrix, act as a protective housing for the microbial community, and work to establish biofilm dynamics (Høiby et al., 2010). Biofilms are now generally considered to be common to many bacterial species, in both natural settings and during infection (McDougald et al., 2011). Given their extensive prevalence, bacterial biofilms have important implications in the medical world, notably those produced by *Pseudomonas aeruginosa* in lung infections of patients with cystic fibrosis (CF), and those produced by *Staphylococcus aureus* in infected wounds and in the case of implanted medical devices (Bjarnsholt et al., 2009; Yarwood et al., 2004).

Adhesion of planktonic (free swimming) bacteria to a surface is the first step to biofilm development, and frequently occurs in high-flow environments (Donlan & Costerton, 2002; Høiby et al., 2010). This reversible step is mediated by cell-surface adhesins, molecules that facilitate direct and specific binding to receptors or other molecules at the site of attachment. In human tissue, the target molecules are often components of the host extracellular matrix such as collagen or laminin (Nallapareddy et al., 2000). For attachment to a clean substratum, such as the surface of an intravenous catheter, environmental macromolecules form a conditioning film within minutes of substratum immersion, providing the necessary binding site for bacterial adhesins (Marshall, 1992).

This is followed by irreversible attachment of the bacteria, characterized by the loss of flagella, and replication to form microcolonies (Høiby et al., 2010; Prüß et al., 2006). At this stage, EPS secretion begins to form the biofilm matrix, and the colony develops into the mature biofilm.

Dispersal of bacterial cells, both actively through the release of planktonic cells and passively by action of environmental shear forces, allow for dissemination to new sites of colonization (Marshall, 1992; McDougald et al., 2011). The process of biofilm development has been reviewed in depth by Marshall (1992). Quorum sensing, a mechanism of communication and coordination between bacteria is thought to be an important factor in the control of biofilm development. This phenomenon relies on the secretion of chemical signal molecules, autoinducers, which increase in concentration in response to increasing population density. When the threshold concentration is reached, signalling pathways alter gene expression within bacterial cells (Miller & Bassler, 2001). Research using *S. aureus* has identified several quorum sensing systems that may be involved in modulating the formation and dispersal of biofilms, and can act to alter the biofilm's susceptibility to antibiotics (Balaban et al., 2007; Yarwood et al., 2004). The process of biofilm formation is summarized in **Figure 1**.

Figure 1. Developmental stages of a *Pseudomonas aeruginosa* biofilm. 1) Initial reversible attachment of planktonic cells to a surface. 2) Irreversible attachment to the surface, characterized by the loss of flagella, and development of microcolonies. 3) Quorum sensing and gene activation leads to biofilm maturation through increased proliferation and EPS secretion to form the matrix. 4) As the mature biofilm develops, colony growth slows and biofilm dynamics are established. 5) Dissemination of biofilm cells by environmental shear forces or the release of planktonic cells.

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In clinical settings, bacterial biofilms have been associated with increased resistance to antibiotics and disinfectants when compared to planktonic cells (Chuard et al., 1991; Oie et al., 1996). This results in persistent or chronic infections, which are difficult to clear both by the host immune system and through traditional treatment mechanisms, and can lead to increased mortality (Costerton et al., 2003; Donlan & Costerton 2002). This review discusses the inherent mechanisms of antibiotic resistance associated with bacterial biofilms, and how this resistance contributes to the progression of disease in clinical settings.

Antibiotic Penetration

Perhaps the simplest explanation to increased antibiotic resistance observed in biofilms is the slow or incomplete penetration of the antibiotic through the EPS matrix. Experimental results on this subject are contradictory, and as such the topic has been heavily debated. Using mathematical modeling, Stewart (1996) theorized the relative penetration of several molecules through the biofilm. The work concluded that most molecules were capable of readily penetrating biofilms, with a 40% diffusion coefficient compared to pure water. Though penetration was slowed, this alone was insufficient to explain the observed antibiotic resistance (Stewart, 1996). While the EPS secretions are complex in nature, the matrix is primarily composed of water, and indeed its main function is the retention of water to hydrate the biofilm (Flemming & Wingender, 2010). This potential decrease in diffusion rate was also observed in experimental studies with glycoprotein and polysaccharide gels, which effectively mimic the diffusion of molecules through the biofilm matrix (Stewart, 2002).

Using *in vitro* models to study antibiotic permeation against biofilm-forming bacteria, several groups have determined that observed penetration is highly dependent on the individual antibiotic (Stewart, 2002). Shigeta et al. (1997) investigated the permeation of various antibiotics through *P. aeruginosa* biofilms grown for zero, one, three, and five days. It was found that the permeation rate of fluoroquinolones was 100% at all time points, whereas aminoglycosides dropped to a permeation rate of 25% after just one day of biofilm growth (Shigeta et al., 1997). These findings were reproduced by Walters III, Roe, Bugnicourt, Franklin, and Stewart (2003), whose study demonstrated a decreased permeation of tobramycin, an aminoglycoside, but not ciprofloxacin, a fluoroquinolone, in *P. aeruginosa* biofilms compared to control groups grown under the same conditions. The biofilm in the Walters III et al. (2003) study was eventually saturated with tobramycin; however, it showed no major difference in antibiotic resistance compared to biofilms treated with ciprofloxacin.

The combined results suggest that under some conditions the matrix can act as a molecular sieve to retard antibiotic permeation; however, this alone does not seem enough to account for the antibiotic resistance inherent in bacterial biofilms. With extended treatment, the biofilm is eventually saturated with antibiotic yet continues to maintain its high level of antibiotic resistance. One possibility is that decreased antibiotic permeation could work to increase the activity of adaptive cellular resistance mechanisms (reviewed later in this article). In support of this hypothesis, *Klebsiella pneumonia* biofilms formed by wild-type strains were found to be resistant to ampicillin saturation, while ampicillin readily penetrated biofilms formed by β -lactamase-negative mutants. Nonetheless, β -lactamase-negative biofilms treated with $1.8 \mu\text{g mL}^{-1}$ of ampicillin maintained an increased resistance to the antibiotic compared to planktonic cells

(MIC of $0.18 \mu\text{g mL}^{-1}$), indicating that another mechanism must also underlie bacterial resistance (Anderl et al., 2000).

Altered Microenvironments

The matrix of a mature biofilm is far from a homogeneous environment. Gradients of nutrients and waste products establish chemically distinct microenvironments throughout the matrix. Nutrients that are readily consumed are taken up by the bacteria closest to the biofilm-fluid interface and cannot easily penetrate to the inner core of the biofilm. Conversely, waste products tend to accumulate at the core of the biofilm, where exchange with the external fluid is limited (Stewart & Franklin, 2008). The best studied example of this phenomenon is with oxygen concentrations through a biofilm. Environmentally-supplied oxygen is quickly consumed by bacteria in the peripheral biofilm, creating anoxic zones close to the center (Costerton et al. 1995). In multi-species biofilms, this results in an accumulation of aerobic bacteria near the peripheries, and anaerobic bacteria in the anoxic zones. However, single species biofilms with facultatively anaerobic bacteria such as *P. aeruginosa* show the same oxygen distribution, with aerobic metabolism in the peripheries and fermentative metabolism occurring in the anoxic zones (Costerton et al., 1995; Marshall, 1992). One consequence of establishing oxygen gradients is that pH gradients are also established through asymmetric metabolism within the biofilm. With aerobic metabolism at the biofilm peripheries and fermentation in the core, there is a marked decrease in pH mirroring the decrease in oxygen concentrations (Vroom et al., 1999).

The activities of many common antibiotics are altered by poor oxygen levels. In one study, *Escherichia coli* entrapped in artificial biofilm-like structures displayed greater resistance to aminoglycosides and β -lactam antibiotics when grown anaerobically versus aerobically (Tresse et al., 1995). These results were repeated in young *P. aeruginosa* biofilms, where resistance against a wide range of drugs, including fluoroquinolones and β -lactam antibiotics, was significantly greater in anaerobically grown biofilms than their aerobic counterparts (Borriello et al., 2004). Changes in pH throughout the biofilm may also impact the efficacy of antibiotics, though research suggests that the observed effect is highly dependent on the drug in question. The efficacy of ampicillin (a β -lactam antibiotic) on isolates of methicillin-resistant *S. aureus* (MRSA), for example, improved as the pH was decreased from 7 to 5.5, while the opposite effect was observed for clindamycin (a lincosamide antibiotic) where the same drop in pH resulted in a decrease in antibiotic effectiveness (Thomas et al., 2011).

As a consequence of decreased pH and oxygen concentration in the biofilm core, the growth rate of cells can be dramatically slowed, especially in single-species biofilms where mechanisms of anaerobic metabolism are not favoured

(Stewart & Franklin, 2008). Analysis of *S. aureus* gene expression during biofilm development showed high cellular activity along the biofilm peripheries, and reduced cellular activity towards the core. In mature biofilms, sessile yet viable cells are present throughout the extent of the biofilm, and may provide scaffolding for biofilm repair when metabolically active cells are lost (Yarwood et al., 2004). Many classes of antibiotics specifically target actively growing cells; for example, β -lactam antibiotics interfere with peptidoglycan synthesis. The slow growth associated with sessile cells in both developing and mature biofilms serves to dramatically increase antibiotic resistance, while not limiting the viability of cells (Anwar & Costerton, 1990).

Phenotypic Variation and Mutations

Bacteria in biofilms have long been known to diversify into a wide range of physiological states, based largely upon adaptation to altered microenvironments. Building on this concept, one consistent phenomenon is the presence of persister cells, highly resistant cells that display multi-drug tolerance (Lewis, 2006). Stochastic gene switching, the randomized switching of cells between various physiological states, is one proposed mechanism to explain the presence of persister cells in biofilm populations (Stewart & Franklin, 2008). Evidence for this is apparent when comparing the survival of bacteria in microcolonies and early developing biofilms with not only antibiotics, but also detergents and disinfectants. In their early stages, the biofilms are too thin to alter antibiotic permeation or develop chemical microgradients (Marshall, 1992; Stewart, 2002). As such, resistance is not comparable to that of a mature biofilm; however, it is still greater than in planktonic cells (Cochran et al., 2000). On their own, persister cells recovered from mature biofilms demonstrate increased antibiotic resistance compared to their planktonic counterparts (Brooun et al., 2000). The presence of persister cells may also contribute to the survival of biofilms after extended antibiotic treatment. *S. aureus* infections have been known to spontaneously relapse in patients with non-healing wounds, even with extensive antibiotic treatment where the biofilm appears to have been cleared entirely (Percival et al., 2011). Persister cells can survive long antibiotic treatments that would kill most other biofilm cells, and can return to a rapidly growing state when the antibiotic is removed (Stewart & Franklin, 2008).

Bacteria growing in biofilms have been shown to have dramatically increased rates of mutation and horizontal gene transfer compared to planktonic cells (Driffield et al., 2008; Molin & Tolker-Nielsen, 2003). These mutations present a significant source of increased antibiotic resistance through alteration of the antibiotic target. A direct mutation in *gyrA* of DNA gyrase, for example, is one proposed mechanism of resistance to quinolone activity that occurs by altering the antibiotic's target (Fàbrega et al., 2009). Horizontal gene transfer, meanwhile, further increases the development of

antibiotic resistance by transferring resistance genes from one cell or group of cells in a biofilm to another. This process has been researched extensively and was deemed a driving force behind the emergence of multi-drug resistance in the nosocomial setting, which involves pathogens such as MRSA and vancomycin-resistant *Enterococcus* (VRE) (Dzidic & Bedekovic, 2003). VRE isolates contain a plasmid-encoding VanA, which is responsible for high levels of vancomycin resistance. In multispecies biofilms made up of both VRE and MRSA, this plasmid can be transferred to MRSA isolates, creating a subpopulation of vancomycin-resistance *S. aureus* (VRSA). In addition to the drug resistance characteristic of MRSA, VRSA is also resistant to vancomycin, the previous treatment of choice for highly-resistant Staphylococcal infections (Weigel et al., 2007). The hypermutable state of biofilms means that multi-drug resistance can easily develop in a single cluster of cells, and spread through the extent of the biofilm via conjugation and transformation. Hypermutable forms of *P. aeruginosa* are routinely isolated from the lungs of CF patients, and could represent a key factor in the persistence of CF-associated infections (Driffield et al., 2008; Høiby et al., 2010).

Adaptive Resistance Mechanisms

Classical cellular mechanisms of antibiotic resistance are conserved in biofilms. As mentioned previously, the activity of these adaptive resistance mechanisms can be increased by biofilm structure. In broad terms, these classical resistance mechanisms can be described as any action that: (a) alters the antibiotic target (as in the *gyrA* mutation discussed above); (b) degrades the antibiotic (such as chromosomal β -lactamase production); or, (c) prevents the antibiotic from reaching its target (Høiby et al., 2010). Chromosomal resistance genes such as those encoding β -lactamase in *P. aeruginosa*, were shown to be upregulated when the system was exposed to relevant antibiotics (Giwerzman et al., 1991). The release of β -lactamase into the matrix may have acted to retard the penetration of penicillins into the biofilm by enzymatically cleaving the target antibiotic, and likely conferred resistance to bacteria part of a multi-species biofilm that may not have otherwise contained the relevant resistance genes (Høiby et al., 2010).

Additionally, *E. coli* cells exposed to antibiotics were shown to have considerable alterations in their outer membrane protein composition, notably a reduction in membrane porins. This response could potentially limit the cellular uptake of antibiotic compounds, and increase resistance for the organism (Mah & O'Toole, 2001). Genetic screening of *P. aeruginosa* isolates, capable of forming biofilms but with significantly reduced antibiotic resistance, revealed the synthesis of periplasmic-localized glucans. These glucans interacted physically with tobramycin, sequestering the antibiotic and preventing it from reaching its target (Mah et al., 2003).

Another adaptive response mechanism is the upregulation of multi-drug efflux pumps, transporters that unspecifically pump antibiotics and antimicrobial agents out of cells. These efflux pumps are generally expressed constitutively in low numbers, and as with β -lactamase may be upregulated in response to challenge with antibiotic (De Kievit et al., 2001; Islam et al., 2009; Pamp, Gjermansen, Johansen, Tolker-Nielsen, 2008; Poole & Srikumar, 2001). The major classes of efflux pumps involved with drug resistance are reviewed by Piddock (2006). The action of many systems is shown to target both antibiotics and natural host-defence molecules, highlighting the ability of efflux pumps to increase biofilms resistance to innate host immune defenses and making them a further concern in clinical settings (Piddock, 2006).

A potential and often overlooked resistance mechanism in Gram-negative organisms is the production of outer membrane vesicles (OMVs). OMVs are bilayered extrusions of the Gram-negative outer membrane, which are constantly discharged from the cell during growth. As these vesicles are released, components of the periplasm are entrapped; thus, OMVs contain large amounts of periplasmic components. The OMV membrane itself retains the typical bilayer structure of the parent cell: an asymmetric lipid-based membrane with LPS on the outer leaflet, and a high number of membrane proteins (Beveridge, 1999; Schooling & Beveridge, 2006). In biofilms derived from Gram-negative or mixed-species bacteria, OMVs are a large component of the matrix, and are associated with diverse functions such as proteolytic activity and antibiotic binding (Schooling & Beveridge, 2006). Proteolytic activity is best seen with β -lactamase packaged into OMVs, and is thought to be the major source of matrix-localized β -lactamase in relevant biofilms (Ciofu et al., 2000). Since the membrane vesicles contain porins from their outer-membrane precursor, β -lactam antibiotics can readily diffuse into the OMV where they are degraded (Beveridge, 1999). During their release from the parent cell, OMV binding of antibiotics allows for cellular "detoxification," removing drugs bound to the outer membrane surface such as polymyxin B and other membrane-disrupting drugs (Manning & Kuehn, 2011). Likewise, β -lactam antibiotics and drugs localized to the periplasmic space could also be encapsulated and released from the parent cell. While this mechanism of detoxification provides the cell with immediate protection against a drug, it does not induce lasting resistance, nor will it confer protection to neighboring cells (Beveridge, 1999; Kuehn & Kesty, 2005; Manning & Kuehn, 2011).

Conclusion

Biofilms are increasingly being regarded as important in clinical settings and in the onset of disease. The high levels of drug resistance that are characteristic to biofilms can be attributed to several factors: inhibited antibiotic penetration, altered microenvironments, phenotypic variations, and adaptive resistance mechanisms. While this review demonstrates that the relative contribution of each mechanism may vary, together they contribute to conferring considerable antibiotic resistance to cells within the biofilm. It is clear that two model organisms are commonly used in the study of bacterial biofilms: *S. aureus*, and *P. aeruginosa*. In clinical settings, these two organisms contribute to a large portion of biofilm-implicated infectious disease. However, current diagnostic and treatment methods focus on planktonic cells and do not adequately represent the biofilm-localized cells of many chronic bacterial infections. As such, while *in vitro* testing may show high antibiotic susceptibility of the pathogen to a particular drug, these results may not always correlate *in vivo*. Expanding our current understanding of how resistance mechanisms function and how they can be overcome is essential to developing new and much-needed techniques to treat infections caused by bacterial biofilms.

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