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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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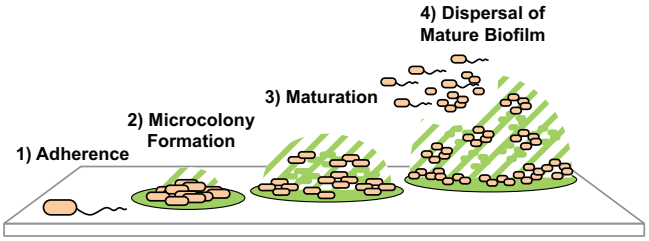

Fig. 1. Biofilm formation progresses through 4 general phases of development. (1) Initial attachment of a cell to a surface is the starting point for the formation of a biofilm. Changes in gene expression lead to the down-regulated expression of polar flagella and up-regulated expression of Type IV pili. 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