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Interdependence between iron acquisition and biofilm formation in *Pseudomonas aeruginosa*

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Abstract

Bacterial biofilms remain a persistent threat to human health-care due to their role in the development of antimicrobial resistance. To combat multi-drug resistant pathogens, it is crucial to enhance our understanding of not only the regulation of biofilm formation, but also its contribution to bacterial virulence. Iron acquisition lies at the crux of these two subjects. In this review, we discuss the role of iron acquisition in biofilm formation and how hosts impede this mechanism to defend against pathogens. We also discuss recent findings that suggest that biofilm formation can also have the reciprocal effect, influencing siderophore production and iron sequestration.

Keywords

iron acquisition; biofilm; nutritional immunity; siderophore; exopolysaccharides; *Pseudomonas aeruginosa*

Introduction

Probably due to the common experience of growing lab-adapted strains of bacteria in liquid cultures, we tend to imagine most bacterial growth (both in the environment and during infection) as taking place in a planktonic state, where individual cells interact with each other through quorum sensing systems (although this perception is also inaccurate, as biofilm aggregates frequently exist in liquid cultures as well [Kragh *et al*., 2018]). In reality, most bacterial growth takes place in the context of biofilms, where structured communities of one or more microbial species are surrounded by extracellular polymeric substances (EPS), including polysaccharides, extracellular DNA, and polypeptides (Friedman and Kolter, 2004; Mulcahy *et al*., 2008; Flemming and Win-gender, 2010). In *Pseudomonas aeruginosa*, biofilm development follows a stereotypical course (Sauer *et al*., 2002; Stoodley *et al*., 2002). This process is generally broken into five different stages: initial attachment, irreversible attachment and EPS secretion, initial development of biofilm architecture, maturation of biofilm architecture, and “shedding” of single cells from mature biofilms.

Biofilms are ancient (they represent the oldest fossils that have been discovered on the planet [Rasmussen, 2000]) and they form complex, heterologous structures (Bridier *et al*., 2010). Some even include channels that permit the diffusion of nutrients and oxygen throughout the

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biofilm, facilitating growth (Kim and Lee, 2016). Several hypotheses have been advanced about the evolutionary origins and advantages of biofilm growth and have suggested that it may have arisen as a mechanism to resist transient shear forces in fluid flow (Rittman, 1982; Peyton and Characklis, 1993; Peyton, 1996), to increase the effective concentration of nutrients and signaling molecules in the proximity of the biofilm (Donlan, 2002), to resist noxious environments (Hall-Stoodley *et al.*, 2004), or even simply to help the bacteria occupy an available area for growth.

Although their evolutionary origins remain speculative, the biomedical consequences of biofilm formation are clear. For several reasons, biofilms dramatically increase resistance to antimicrobials. First, they enrobe the bacteria in EPS, limiting the diffusion of biocides and antibacterials and their access to cells (Mah and O'Toole, 2001; Anderson and O'Toole, 2008). For example, a number of studies have shown a correlation between biofilm viscosity and antimicrobial sensitivity (Stewart, 1996; Gilbert *et al.*, 1998; Wirtanen *et al.*, 1998; Kostenko *et al.*, 2007; Ruhs *et al.*, 2013). Genetic disruption or chemical inhibition of biofilm formation also increases pathogen susceptibility to antimicrobial agents (Rashid *et al.*, 2000; Shih and Huang, 2002; Li and Lee, 2017). Biofilms are also thought to reduce the growth rate of bacterial cells within their matrix, facilitating the appearance of morphologically-distinct cells commonly called 'persisters', which show uncommonly high tolerance to insults (Drenkard and Ausubel, 2002; Kester and Fortune, 2014). Phenotypically normal cells can also exhibit diminished antimicrobial sensitivity when their rate of cell division decreases, a phenomenon sometimes called antimicrobial indifference (Jayaraman, 2008). Biofilm formation also promotes the evasion of host immune recognition, phagocytosis, and host bacterial killing (Jensen *et al.*, 1990; Costerton *et al.*, 1999; Leid *et al.*, 2005; Alhede *et al.*, 2014).

As antimicrobial resistance continues to mount, biofilms have become an important therapeutic target in the treatment of infectious diseases. Disruption of established bio-films promotes the removal of bacteria by the immune system and conventional antimicrobials and limits other bio-film-dependent mechanisms of virulence. Small molecules and genetic mutations that compromise biofilm formation have been shown to limit pathogenesis (Hentzer *et al.*, 2003; Cady *et al.*, 2012; Komor *et al.*, 2012; O'Loughlin *et al.*, 2013; Kang and Kirienko, 2017), demonstrating their utility.

Biofilm formation is an integral part of *P. aeruginosa* infection in mammals, especially in the airways of patients with cystic fibrosis (CF), where this pathogen is a leading cause of death (Singh *et al.*, 2000; Winstanley *et al.*, 2016; Moradali *et al.*, 2017). Despite their obvious value, developing therapeutics that target *P. aeruginosa* biofilms is challenging. Biofilm formation in *P. aeruginosa* is particularly complex, involving a variety of redundant regulatory mechanisms (Colvin *et al.*, 2012; Irie *et al.*, 2012). For example, both intracellular and intercellular signaling via secondary messengers such as cyclic diguanylate monophosphate (c-di-GMP) and quorum sensing molecules such as homoserine lactones, are involved in the 'decision' to begin the transition to sessile development (Parsek and Greenberg, 2000; Goodman *et al.*, 2004; Camilli and Bassler, 2006; Sakuragi and Kolter, 2007; Petrova and Sauer, 2009; Mikkelsen *et al.*, 2011). As a consequence, various studies

have proposed an alternative approach to mitigating biofilm formation: prevent the pathogen from acquiring sufficient iron, and the process will be compromised.

Iron acquisition is necessary for biofilm formation in *P. aeruginosa*

In a seminal study, Singh and colleagues reasoned that if bio-films were such a common occurrence in human infections, the innate immune system should mount some defense against them (Singh *et al.*, 2002). Through careful study they discovered that lactoferrin sequestered iron from the pathogen, causing iron deprivation. This state induced bacterial twitching, preventing the formation of microcolonies. Further works by Greenberg and colleagues showed that pyoverdine (a key siderophore produced by *P. aeruginosa*, see below) was necessary for the development of biofilms *in vitro* (Banin *et al.*, 2005, 2006). In contrast, loss of the other major siderophore, pyochelin, had no effect on biofilm development. They also observed that supplementation of the media with ferric citrate (a salt that is actively transported into the bacterium) restored biofilm formation in the absence of pyoverdine, suggesting that iron transportation was the relevant determinant, rather than pyoverdine *per se*. Based on these observations, a number of studies tested the impact of synthetic iron chelators (such as deferasirox, ethylene diamine tetraacetic acid, ethylene diamine-*N,N′*-bis(2-hydroxyphenylacetic acid, and others) on *P. aeruginosa* biofilm formation (Banin *et al.*, 2006; Moreau-Marquis *et al.*, 2009; O'May *et al.*, 2009; Kang and Kirienko, 2017) (Fig. 1). In each case, these compounds disrupted the formation of biofilms.

Bacterial iron uptake can also be compromised by gallium, which exists as a trivalent cation with an ionic radius and charge density comparable to iron (III) (Kaneko *et al.*, 2007). Unlike iron, however, gallium is redox inactive, generally precluding it from carrying out the same biochemical functions as iron (Chitambar and Narasimhan, 1991). As such, it is perhaps unsurprising that gallium nitrate killed multi-drug resistant *P. aeruginosa*, prevented biofilm formation, destroyed established biofilms, and effectively mitigated *P. aeruginosa* pathogenicity in multiple murine infection models (Kaneko *et al.*, 2007; Banin *et al.*, 2008). It is worth noting that several clinical trials utilizing gallium salts as inhibitors of bacterial infection are currently underway. Since gallium nitrate is already an FDA approved drug (for treatment of hypercalcemia), the pathway to repurposing gallium nitrate as an infection therapeutic is simpler than the development of previously unidentified compounds.

Interestingly, anaerobic growth of *P. aeruginosa* (such as the conditions believed to exist in the mucosal secretions of cystic fibrosis patients' airways [Worlitzsch *et al.*, 2002]) is known to stimulate the development of thick, robust bio-films (Yoon *et al.*, 2002). For reasons that remain unclear, growth and biofilm formation under anaerobic conditions demands higher concentrations of ferric iron than otherwise, making *P. aeruginosa* more susceptible to growth inhibition by chemical chelators (O'May *et al.*, 2009). Interestingly, the most common causative mutation for CF (the ΔF508 mutation, which refers to the loss of the phenylala-nine at position 508 in the CFTR protein) triggers the secretion of excess iron into the extracellular milieu, creating conditions that favor biofilm formation. When *P. aeruginosa* was grown on a cell line derived from a patient homozygous for the ΔF508 mutation, the increase in extracellular iron caused by this mutation significantly enhanced biofilm production (Moreau-Marquis *et al.*, 2008). Much as in other anaerobic or

microaerobic conditions, these conditions showed increased sensitivity to iron chelators, specifically conalbumin, which strongly limited biofilm formation (Bernardini *et al.*, 1993; Hunter *et al.*, 2013).

It should be noted that the phenomena described thus far are likely only to apply to biofilms that are comprised of Pel or Psl. Alginate, the third biofilm polysaccharide produced by *P. aeruginosa*, is overproduced in mucoid strains isolated from the respiratory tracts of cystic fibrosis patients due to one or more mutations in *muc* genes (Franklin *et al.*, 2011). Unlike Pel or Psl, alginate is not attached to the cell's surface, but is secreted into the extracellular milieu. Surprisingly, not only is iron dispensable for alginate biosynthesis, but iron-replete conditions appear to limit alginate production. For example, mucoid strains grown in the presence of iron are unstable and prone to being supplanted by non-mucoid strains (Boyce and Miller, 1980, 1982). In addition, iron starvation can stimulate the appearance of mucoid strains (Terry *et al.*, 1992). However, Vasil and colleagues have noted that at least some mucoid strains of *P. aeruginosa* appear to have lost the ability to regulate alginate production in iron-replete conditions (Oglesby-Sherrouse *et al.*, 2014).

Nutritional immunity: host inhibition of biofilm formation

The idea of limiting bacterial access to iron is not new. In fact, it could be said that the idea is at least 400 million years old. That is the estimated date of the divergence of the Euteleostomi, amongst most members of which ferritin and transferrin are conserved. In a broader sense, hosts and pathogens compete for several bioavailable transition metals (including iron, copper, zinc, manganese, and molybdenum) (Hood and Skaar, 2012). These metals are required for gene transcription, redox-reactions, and even non-redox, metal-dependent reactions (such as the prolyl hydroxylase domain [PHD]-containing family of proteins that use ferrous iron to split molecular oxygen for protein hydroxylation). The process whereby the host restricts access to these metals is colloquially known as nutritional immunity and has been recently reviewed elsewhere (Palmer and Skaar, 2016; Carver, 2018), so our attention will focus on iron.

To prevent pathogens from acquiring this essential nutrient, hosts withhold intracellular iron using iron-storage proteins such as ferritin or in iron-containing complexes like heme, and restrict extracellular iron availability by secreting iron-sequestering proteins such as transferrin and lactoferrin (Skaar, 2010; Kelson *et al.*, 2013). Transferrin and lactoferrin function similarly to chemical iron chelators; by restricting environmental iron, bacterial biofilm formation is compromised (Fig. 2). For example, apo-transferrin significantly attenuates biofilm formation in *Staphylococcus epidermidis* and attachment in *S. aureus* and *P. aeruginosa* (Ardehali *et al.*, 2002; She *et al.*, 2016). The removal of apotransferrin (or replacing it with iron-saturated transferrin) permits re-establishment of biofilm formation and bacterial adhesion in these systems. Similar results are observed when bacteria are treated with apo-lactoferrin (Singh *et al.*, 2002; Banin *et al.*, 2005; Wakabayashi *et al.*, 2009; Kamiya *et al.*, 2012).

Pathogens attempt to overcome iron limitation in at least three ways. First, some pathogens express receptors for lactoferrin or transferrin, in a bid to acquire the proteins and their

associated iron (Beddek and Schryvers, 2010; Pogoutse and Moraes, 2017). Second, many human pathogens have heme acquisition pathways comprised of heme-binding receptors and/or even heme-binding molecules called hemophores (Cescau *et al.*, 2007; Huang and Wilks, 2017). Finally, and most commonly, most pathogenic and many non-pathogenic species of bacteria (and fungi) produce small molecule iron chelators called siderophores. These molecules have been evolved to improve the aqueous solubility of iron (III). To facilitate their biological role, these molecules have exceptionally high affinities to ferric iron. This also helps them overcome host iron restriction mechanisms by directly chelating ferric iron from host iron-sequestering proteins (Skaar, 2010) (Fig. 2). For instance, both enterobactin (a high-affinity siderophore produced by a variety of Enterobacteriaceae, including *Escherichia coli* and *Salmonella typhimurium*) and pyoverdine (produced by *P. aeruginosa*) can acquire iron from human iron storage proteins such as transferrin or ferritin (Kvach *et al.*, 1977; Guterman *et al.*, 1978; Carrano and Raymond, 1979; Harris *et al.*, 1979; Tidmarsh *et al.*, 1983; Wolz *et al.*, 1994; Meyer *et al.*, 1996; Xiao and Kisaalita, 1997).

To inhibit siderophore activity, mammalian hosts secrete the siderophore binding protein lipocalin 2 (also known as neutrophil gelatinase-associated lipocalin, or NGAL, to differentiate it from lipocalin 1, which is derived from tears) to recognize and bind to siderophores such as enterobactin (Fig. 2) (Goetz *et al.*, 2002; Flo *et al.*, 2004). Lipocalin 2 is critical for innate immunity, as lipocalin 2-deficient mice exhibit increased bacteremia and sepsis during infection with *E. coli* (Flo *et al.*, 2004; Berger *et al.*, 2006). *In vitro*, when bacteria are grown in iron-limited media, lipocalin 2 treatment has a growth-inhibitory and antivirulent effect, which is mitigated by the supplementation of enterobactin or ferrichrome (as a source of iron), suggesting that lipocalin 2 rescues hosts by depriving the pathogen of iron (Flo *et al.*, 2004). However, while lipocalin 2 production has been shown to be an effective host immune response against some pathogens, others (including *Klebsiella pneumoniae*, *Salmonella enterica*, and *P. aeruginosa*) have evolved mechanisms to circumvent this defense. For example, lipocalin 2 does not efficiently bind pyoverdine (Peek *et al.*, 2012), while *K. pneumoniae*, *E. coli*, *and S. enterica* can evade lipocalin 2 by secreting a glycosylated version of enterobactin known as salmochelin (Fischbach *et al.*, 2006). It is worth pointing out that immature or improperly-folded salmochelin molecules can be bound by lipocalin 2 (Valdebenito *et al.*, 2007). *K. pneumoniae* and *Yersinia* species also produce a structurally unrelated siderophore called yersiniabactin, which is also not affected by lipocalin 2 (Bachman *et al.*, 2011). Notably, the presence of both enterobactin and yersiniabactin is associated with successful colonization of the respiratory niche by strains of *K. pneumoniae* (Bachman *et al.*, 2011).

Biofilm formation promotes siderophore production

As noted previously, iron acquisition is necessary for the proper development of biofilms by *P. aeruginosa*. In many cases, this need is fulfilled by pyoverdine, inextricably linking these two secreted products.

Pyoverdine is arguably the most important siderophore in the *in vivo* growth and pathogenesis of *P. aeruginosa*, as demonstrated by the avirulence of pyoverdine-deficient mutants in a variety of infection models (Meyer *et al.*, 1996; Takase *et al.*, 2000; Kirienko *et*

al., 2013; Minandri *et al.*, 2016). Historically, this has been attributed to its ability to also function as a determinant for the activity of the alternate sigma factor PvdS, which controls the expression of several secreted virulence factors, including the translational inhibitor ToxA and the protease PrpL (Lamont *et al.*, 2002). However, more recent data suggest that pyoverdine may also damage host mitochondria by removing iron, triggering mitochondrial turnover (Kirienko *et al.*, 2015; Kang *et al.*, 2018).

Due to its clinical significance, we carried out a high-throughput screen to identify genes necessary for pyoverdine bio-synthesis (Kang and Kirienko, 2017). Surprisingly, this screen yielded many components of biofilm formation, such as Pel exopolysaccharide, flagella, and type IV pili. It is important to note that this phenomenon was observed under iron-replete conditions (as demonstrated by the ability of pyoverdine-deficient mutants to form wild-type levels of biofilm), suggesting that the impairment of biofilm mitigates pyoverdine production. We hypothesize that this phenomenon may have clinical importance in the respiratory tracts of CF patients, where iron concentrations are known to increase (Hunter *et al.*, 2013) and biofilms often appear (Singh *et al.*, 2000; Winstanley *et al.*, 2016; Moradali *et al.*, 2017). In addition, the ability of *P. aeruginosa* to produce siderophores under iron replete conditions may promote previously unappreciated bacterial proliferation and pathology.

Although the mechanism of biofilm-mediated regulation of pyoverdine remains unclear, there is a strong correlation between biofilm formation and cell aggregation (Visaggio *et al.*, 2015; Kang and Kirienko, 2017). This was initially identified and investigated by Imperi and colleagues, who demonstrated that the exopolysaccharides Pel and Psl were essential for planktonic cell aggregation and pyoverdine production (Visaggio *et al.*, 2015). Artificially inducing cell aggregation, by adding agar to the media for example, was shown to restore pyoverdine in *pel psl* double mutants (Visaggio *et al.*, 2015). Similarly, we observed that the supplementation of the quorum-sensing molecule Pseudomonas quinolone signal (PQS) to media rapidly stimulated aggregation of planktonic cells, causing high levels of pyoverdine (Kang *et al.*, 2017). The addition of PQS also partially restored pyoverdine production in *P. aeruginosa* biofilm mutants (Kang *et al.*, 2017). Together, these findings suggest a model where the aggregation of planktonic cells nucleates biofilm formation and induces the production of pyoverdine in a manner that is separate from its regulation by intracellular iron content.

Sequestration of iron by extracellular matrix components

Another intriguing phenomenon that further complicates the relationship between biofilm, iron, and virulence is the discovery that biofilms can store iron. Although iron is essential for most living organisms, it is also quite toxic at high concentrations as it can catalyze the Fenton reaction, which produces reactive oxygen species (ROS). Therefore, bacteria must maintain a delicate balance, acquiring sufficient iron for growth but not enough to allow the wide-spread production of ROS. It now appears that components of the *P. aeruginosa* biofilm matrix help the bacteria maintain this balance.

Each of the three major polysaccharides produced by *P. aeruginosa* has a different function during biofilm formation. Psl is produced in both planktonic and biofilm cells. In planktonic

cells, Psl promotes cell surface attachment, the initial step of biofilm formation (Ma *et al.*, 2006; Vogeleer *et al.*, 2014). Once cells have attached, Psl exopolysaccharide facilitates biofilm maturation by promoting cell-cell interactions within the extracellular matrix, anchoring the cells to the biofilm (Ma *et al.*, 2006, 2009). In contrast, Pel exopolysaccharide does not play an important role in cell attachment; instead, Pel significantly contributes to biofilm growth by promoting cell-cell interactions necessary for cell aggregation (Colvin *et al.*, 2011). The importance of this function varies across *P. aeruginosa* strains (Colvin *et al.*, 2012). For instance, *P. aeruginosa* PAO1, unlike PA14, primarily utilizes Psl, while Pel is dispensable for biofilm formation (Colvin *et al.*, 2011). Interestingly, this mirrors the importance of this exopolysaccharide on pyoverdine production: while *pel* mutants display attenuated biofilm formation in PA14, they will produce wild-type levels of pyoverdine in PAO1 (Kang and Kirienko, 2017).

A recent study by Ma and colleagues demonstrated that all three of these exopolysaccharides can sequester free environmental iron (Fig. 3). In brief, alginate binds ferric iron, Pel binds ferrous iron, and Psl binds to both (Yu *et al.*, 2016). Importantly, *P. aeruginosa* is capable of utilizing iron bound to Psl to support its growth during in iron-limiting environments (Yu *et al.*, 2016).

This ability to sequester iron is not unique to polysaccha-rides from *P. aeruginosa*. Exopolysaccharides from *Xanthomonas campestris*, *Paracoccus zeaxanthinifaciens*, and *Klebsiella oxytoca* have also been shown to bind iron (Baldi *et al.*, 2009; Moppert *et al.*, 2009; Javvadi *et al.*, 2018). Like Psl, cyclic β-(1,2) glucans from *X. campestris* can store iron that is utilized by the bacteria to support growth under iron-restricted conditions (Javvadi *et al.*, 2018). This phenomenon is wide-spread enough that exopolysaccharides are being considered as potential substrates of heavy metal bioremediation due to their ability to bind various metals (De Philippis *et al.*, 2011; Gupta and Diwan, 2017; Mohite *et al.*, 2017).

Another component of the *P. aeruginosa* biofilm matrix that is capable of sequestering iron is the filamentous bacteriophage Pf4 (Fig. 3). The Pf4 prophage within the *P. aeruginosa* genome is highly expressed in biofilm cells, resulting in orders of magnitude greater phage production in biofilms than planktonic cell cultures (Whiteley *et al.*, 2001; Webb *et al.*, 2004). Pf4 activity is necessary for normal bio-film development and maturation, as well as pathogen virulence (Rice *et al.*, 2009). Phage activity also triggers death of *P. aeruginosa* cells in CF infection isolates (Webb *et al.*, 2003; Kirov *et al.*, 2007), and has been posited to drive *P. aeruginosa* to a mucoid state (Miller and Rubero, 1984; Hoiby *et al.*, 2001). Pf4 bacteriophage in *P. aeruginosa* biofilms can also directly bind to ferric iron, as demonstrated by Raman-binding analysis and the induction of phage cross-linking in the presence of ferric iron (Penner *et al.*, 2016). This iron-chelating activity gives *P. aeruginosa* an advantage during polymicrobial interactions. For instance, *P. aeruginosa* inhibits *Aspergillus fumigatus* biofilm formation via Pf4-mediated iron sequestration (Ferreira *et al.*, 2015; Penner *et al.*, 2016). Pf4 bacteriophage can inhibit *A. fumigatus* biofilms even in the absence of live *P. aeruginosa*, but this inhibition is deterred by supplementation of ferric iron (Penner *et al.*, 2016). Although Pf4 functions as an important component of the *P. aeruginosa* biofilm matrix that can sequester ferric iron, it is currently unknown whether *P. aeruginosa* can utilize iron-bound Pf4 as a source of iron, either directly or indirectly.

Conclusion

Because both host and pathogens require iron for essential cellular processes, iron homeostasis has become a widely studied topic in microbial pathogenesis and immunology. Iron metabolism is involved in many facets of biofilm biology, necessitating the development of pathogen-targeted systems to prevent iron acquisition (e.g., transferrin, lactoferrin, siderophore-binding proteins, etc.) As biofilms enhance bacterial resistance to antimicrobial treatment and facilitate evasion of host immune recognition, they continue to represent an important subject of research.

Based on the competition for iron between hosts and pathogens, many proposals have been made to use a variety of synthetic chelators or gallium to compromise bacterial iron acquisition. However, more recent findings suggest that this approach may be more complicated than initially believed. For example, many biofilm-producing bacterial species grow in polymicrobial communities. It is yet unclear whether disrupting the production of biofilm by a single pathogen in such a community will be clinically beneficial. It may, in fact, be detrimental for host health. For example, iron chelators may affect *P. aeruginosa*, but leave *S. aureus* biofilm production functional, creating an environment where the pathogen can flourish without a competitor for resources. Clearly, a better understanding of host-pathogen, pathogen-pathogen, and other more complex relationships is necessary before we can accurately predict the consequences of tampering with bacterial biofilms or iron acquisition pathways in the context of infection.

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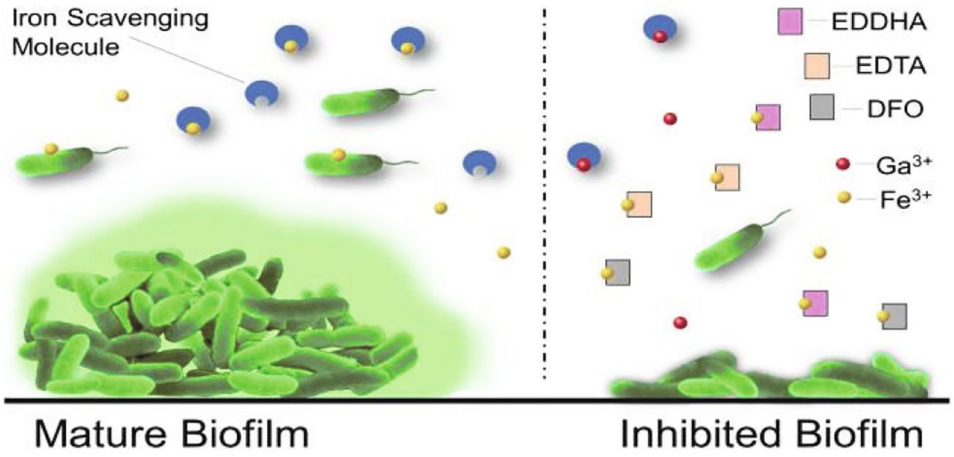

Fig. 1. Interfering with bacterial iron acquisition inhibits biofilm formation.

Under iron-replete conditions, free iron can be either directly transported into the bacterium by active transport systems or indirectly transported via iron scavenging molecules (e.g., pyoverdine, pyochelin, PQS, etc.). Under these conditions bacteria retain the ability to form mature biofilms. In the presence of iron chelators (such as EDTA, EDDHA, or heterologous siderophores that *P. aeruginosa* cannot utilize, like deferoxamine), however, iron availability is restricted and biofilm formation is compromised. This indicates that iron uptake is necessary for biofilm formation. Furthermore, certain metals like gallium can compete against iron for bacterial iron-scavenging molecules, preventing iron uptake and inhibiting biofilm formation.

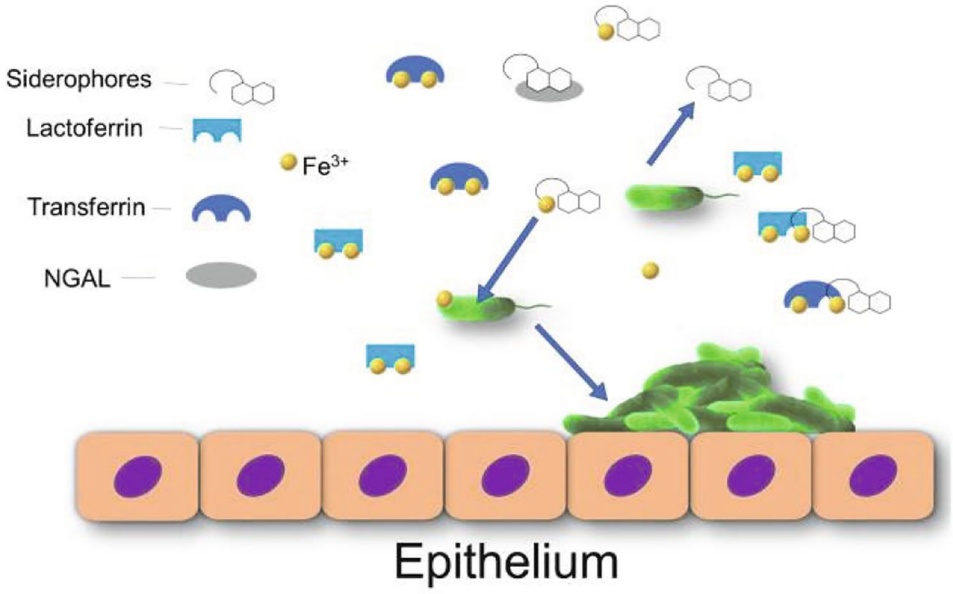

Fig. 2. Hosts and pathogens compete to sequester iron from the environment.

Host cells secrete iron-sequestering proteins such as transferrin and lactoferrin to minimize free extracellular iron. Some bacterial pathogens secrete siderophores to compete against these proteins and scavenge the trace amounts of free iron. Certain siderophores, such as enterobactin (from *E. coli* and *S. typhimurium*) and pyoverdine (from *P. aeruginosa*), can directly remove ferric iron from iron-bound transferrin and lactoferrin, increasing pathogen iron uptake and promoting biofilm formation. To interfere with siderophore activity, certain host cells secrete lipocalin 2 (also known as NGAL) to recognize and bind to siderophores, preventing their function. Production of siderophores that can evade lipocalin 2 has also been linked to pathogenicity.

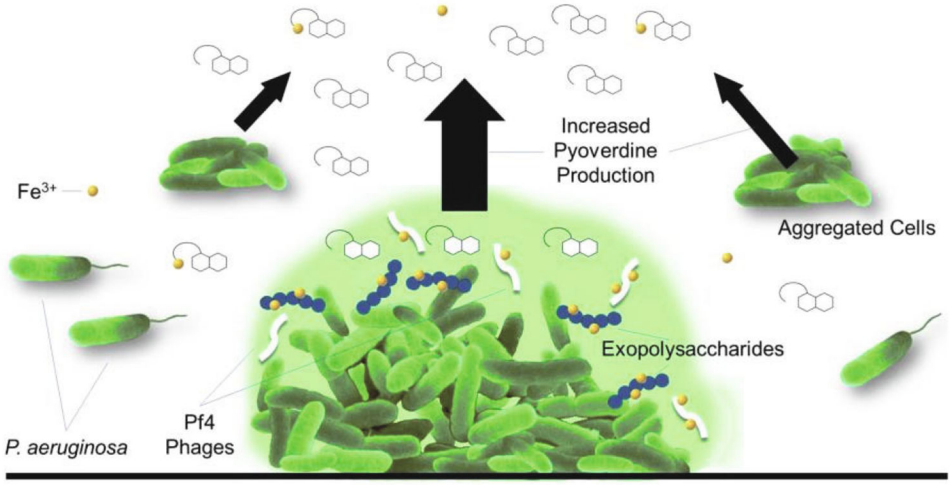

Fig. 3. *P. aeruginosa* biofilm can store excess iron.

In *P. aeruginosa* biofilms, secreted exopolysaccharides and Pf4 bacteriophages can bind iron, sequestering it in the extracellular matrix. Alginate and Pf4 can sequester ferric iron, Pel exopolysaccharide can sequester ferrous iron, and Psl exopolysaccharide can sequester both. In the case of Psl, *P. aeruginosa* can utilize iron-bound Psl for growth and bio-film formation under conditions of iron restriction. Furthermore, aggregated cells (in both planktonic and sessile states) exhibit increased pyoverdine production, suggesting another regulatory mechanism for iron uptake.