Ironing Out the Biofilm Problem: The Role of Iron in Biofilm Formation

E. Peter Greenberg, Ehud Banin

Abstract The opportunistic pathogen *Pseudomonas aeruginosa* causes chronic biofilm-associated infections in the lungs of cystic fibrosis patients that cannot be eradicated by antibiotics. Like most other pathogens, *P. aeruginosa* is under intense competition with the host for iron. Recent studies show that even when there is sufficient iron for growth, this element serves as a signal for biofilm development. Here, we summarize our knowledge of the role iron plays in *P. aeruginosa* biofilm development. Novel therapeutic approaches that target iron homeostasis as an antibiofilm target are also presented.

1 Introduction to the Problem

P. aeruginosa exhibits considerable nutritional and metabolic versatility, which allows it to colonize a wide variety of environments. It can be isolated from soil, water, plants, and animals. Like most organisms, *P. aeruginosa* requires iron for growth, as iron serves as a cofactor for enzymes that are involved in many basic cellular functions and metabolic pathways (Andrews et al. 2003). Recent reports show that iron also serves as an environmental signal for biofilm development (Banin et al. 2005; Singh et al. 2002).

P. aeruginosa causes chronic biofilm-associated infections in the lungs of CF patients (Costerton et al. 1999; Lyczak et al. 2000; Parsek and Singh 2003; Singh et al. 2000). CF is an autosomal recessive disorder resulting from a mutation in a gene coding for a chloride ion channel, the CF transmembrane conductance regulator

E. Peter Greenberg (✉)
School of Medicine, University of Washington, Department of Microbiology, Seattle, WA, USA, e-mail: epgreen@u.washington.edu
Ehud Banin
Bar-Ilan University, The Mina and Everard Goodman Facility of Life Sciences, Ramat Gan, Israel

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(reviewed in Rowe et al. 2005). This single genetic defect has pleiotropic effects on the development and function of several tissues and organs. The mutation results in a hypersusceptibility of CF patients to bacterial infection and to *P. aeruginosa* in particular. Chronic *P. aeruginosa* infection of the lungs currently accounts for the majority of the morbidity and mortality seen in CF (Chernish and Aaron 2003; Davies 2003).

Although the battle to treat and cure CF is far from over, in the last decade a substantial increase in the life span of CF patients has been achieved, mainly due to improvements in antimicrobial therapy and treatment regimes. In fact, in its 2006 patient registry data report, the American Cystic Fibrosis Foundation announced that in the last 4 years alone, more than 5 years have been added to the median survival age of CF patients, which currently stands at 36.8 years. Unfortunately, one must remember that despite this encouraging improvement, half of the patients suffering from CF will not reach this age, reemphasizing the need for novel antimicrobial therapeutic approaches that can effectively eradicate *P. aeruginosa* chronic infection in the lungs.

As an opportunistic pathogen, one of the biggest challenges for *P. aeruginosa* is the acquisition of iron in the host. While iron was readily available and soluble at the beginning of life on earth, the increase of oxygen in the atmosphere as our planet matured dramatically reduced iron solubility and exposed the toxic effects associated with this element due to its ability to mediate the production of oxygen radicals (Touati 2000). Bacteria have developed sophisticated mechanisms to scavenge iron from dilute environments and to maintain iron homeostasis (reviewed in Andrews et al. 2003). It is interesting that as life on earth continued to evolve, the role of iron as an essential element was maintained. In fact, it is so crucial for life that a central part of the mammalian and human host defense against invading pathogens is based on the ability to maintain free iron concentrations at extremely low levels ($< 10^{-18}$ M) (reviewed in Schaible and Kaufmann 2004). Proteins such as lactoferrin and transferrin that tightly bind iron provide an efficient first line of defense against bacterial infections. Thus, there is intense competition for iron with the host, and bacterial acquisition of iron is recognized as one of the key steps in the establishment of an infection (Ratledge and Dover 2000; Schaible and Kaufmann 2004).

Although the role of iron in supporting microbial growth has been known for many years, only recently has it been reported that this metal also serves as a signal for biofilm development. In this chapter we review the current knowledge of how iron mediates biofilm formation, and we discuss the potential of targeting iron uptake and homeostasis as a novel antibiofilm therapeutic approach.

2 Iron Acquisition and Regulation in *P. aeruginosa*

2.1 Iron Uptake

The ability of *P. aeruginosa* to colonize a wide range of environments is, not surprisingly, accompanied by a very sophisticated and versatile iron acquisition sys-

tem. Under iron-limiting conditions, *P. aeruginosa* synthesizes two bacterial ferric ion chelating molecules (i.e., siderophores), pyoverdine and pyochelin (reviewed in Poole and McKay 2003). These siderophores bind ferric ions and transport them into cells via specific outer membrane receptors in a process that is driven by the cytosolic membrane potential and is mediated by the energy-transducing TonB-ExbB-ExdD system (reviewed in Moeck and Coulton 1998). Of the two siderophores, pyoverdine has the higher affinity and can successfully compete for iron with transferrin and lactoferrin (Wolz et al. 1994; Xiao and Kisaalita 1997). Furthermore, mutants defective in pyoverdine synthesis and transport are avirulent in several animal infection models (Meyer et al. 1996; Takase et al. 2000). Interestingly, pyoverdine can also act as a signaling molecule. The binding of ferripyoverdine to the outer membrane receptor FpvA induces, via a cell surface signaling pathway consisting of two extracytoplasmic function (ECF) sigma factors, transcription of its receptor, genes required for the production of pyoverdine, and genes encoding several virulence factors (Beare et al. 2003; Lamont et al. 2002).

Beside its endogenous siderophores, *P. aeruginosa* is also able to utilize a variety of heterologous iron-binding molecules such as heme, enterobactin, ferric citrate, and ferrioxamine to acquire iron (reviewed in Poole and McKay 2003). Two receptors, PhuR and HasR, are involved in the utilization of heme (Ochsner et al. 2002), and two, PfeA and PirA, mediate the uptake of enterobactin (Dean and Poole 1993; Ghysels et al. 2005). Our laboratory has recently shown that *P. aeruginosa* can utilize ferric citrate via a FecI/R-like system (PA3899-3901) and ferrioxamine via two receptors, FoxA (PA2466) and FiuA (PA0470) (Banin et al. 2005). FiuA has also been shown to promote ferrichrome transport (Llamas et al. 2006). It is interesting to note that these receptors probably represent only a small sample of the real iron-acquisition potential of *P. aeruginosa*. An in silico analysis of the *P. aeruginosa* genome revealed an impressive total of 34 genes encoding putative TonB-dependent outer membrane receptors (Cornelis and Matthijs 2002). Combined with the endogenous siderophores, these may give *P. aeruginosa* the ability to "steal" iron from other microorganisms, providing a competitive advantage in the environment.

2.2 Iron Regulation

Because bacteria require iron, which in itself is toxic, cellular iron levels must be tightly regulated. Two independent microarray analyses have revealed that over 200 *P. aeruginosa* genes are differently regulated by iron (Ochsner et al. 2002; Palma et al. 2003). Like many other *Proteobacteria*, iron regulation in *P. aeruginosa* is orchestrated by the ferric uptake regulator (Fur), which appears to be essential for the viability of *P. aeruginosa* (Ochsner et al. 1995; Vasil and Ochsner 1999). Fur binds to specific DNA sequences (Fur boxes) and represses iron-responsive genes. Fur is dependent on intracellular iron because it requires Fe^{2+} as a cofactor for its DNA-binding activity. Although Fur is a repressor, it can also activate expression of specific genes (such as genes coding for iron storage proteins) through its control of a pair of small regulatory RNAs, PrrF1 and PrrF2 (Wilderman et al. 2004).

Fur is not the only regulator that governs the iron response of *P. aeruginosa*. Another group of regulators consists of the iron starvation ECF sigma factors (reviewed in Visca et al. 2002). These environmentally responsive transcription factors enable bacteria to respond to the presence of specific siderophores in the environment as well as to levels of intracellular iron. Visca et al. (2002) utilized the availability of the *P. aeruginosa* genome and identified 14 gene clusters that may encode iron-responsive ECF sigma factor-regulated systems. The best-studied member of this group in *P. aeruginosa* is the ECF sigma factor PvdS, which is known to regulate the synthesis of pyoverdine genes and genes coding for several virulence factors (Beare et al. 2003; Lamont et al. 2002). The combination of Fur, sRNAs, and iron starvation ECF sigma factors provides *P. aeruginosa* a highly regulated system that can efficiently respond to changes in extracellular and intracellular iron concentrations.

3 Iron and *P. aeruginosa* Biofilm Development

3.1 Low Iron – The Lactoferrin Effect

In 2002 Singh and colleagues reported that lactoferrin blocked *P. aeruginosa* microcolony formation on glass surfaces by virtue of its ability to sequester iron (Singh et al. 2002). These authors were intrigued by the ability of healthy hosts to prevent bacterial biofilm formation. They speculated that antimicrobial peptides and proteins produced by mammals might be able to interfere with biofilm development. Mucosal secretions are known to be rich in such antimicrobial factors as lactoferrin, lysozyme, and defensins. The authors' initial study focused on lactoferrin because it is abundant in mucosal secretions. Lactoferrin is a mammalian iron chelator that can limit bacterial growth by sequestering iron. It also has bactericidal activity involving its ability to bind lipopolysaccharides and disrupt membranes (reviewed in Orsi 2004). The effect of subgrowth inhibitory concentrations of lactoferrin on biofilm development was examined (Singh et al. 2002). Using a continuous flow-cell biofilm system as a model to study biofilm development, the authors grew *P. aeruginosa* PAO1 carrying a green-fluorescent-protein-containing plasmid in the presence and absence of lactoferrin. In the absence of lactoferrin, *P. aeruginosa* cells attached and within 48 h formed microcolonies that developed to characteristic mushroom-like structures by 96 h (Fig. 1). In the presence of a subinhibitory concentration of lactoferrin, cells attached and multiplied but failed to form microcolonies and structured biofilms. Rather, they remained as a flat thin layer of cells attached to the glass surface (Fig. 1). Iron-saturated lactoferrin did not cause this inhibition, suggesting that the antibiofilm activity was dependent on lactoferrin's iron chelation activity.

To further investigate the antibiofilm activity of lactoferrin, Singh et al. (Singh et al. 2002; Singh 2004) carried out time-lapse microscopy experiments. They observed that in the absence of lactoferrin, cells attached to the surface and multiplied, and the daughter cells in most cases remained near the point of division.

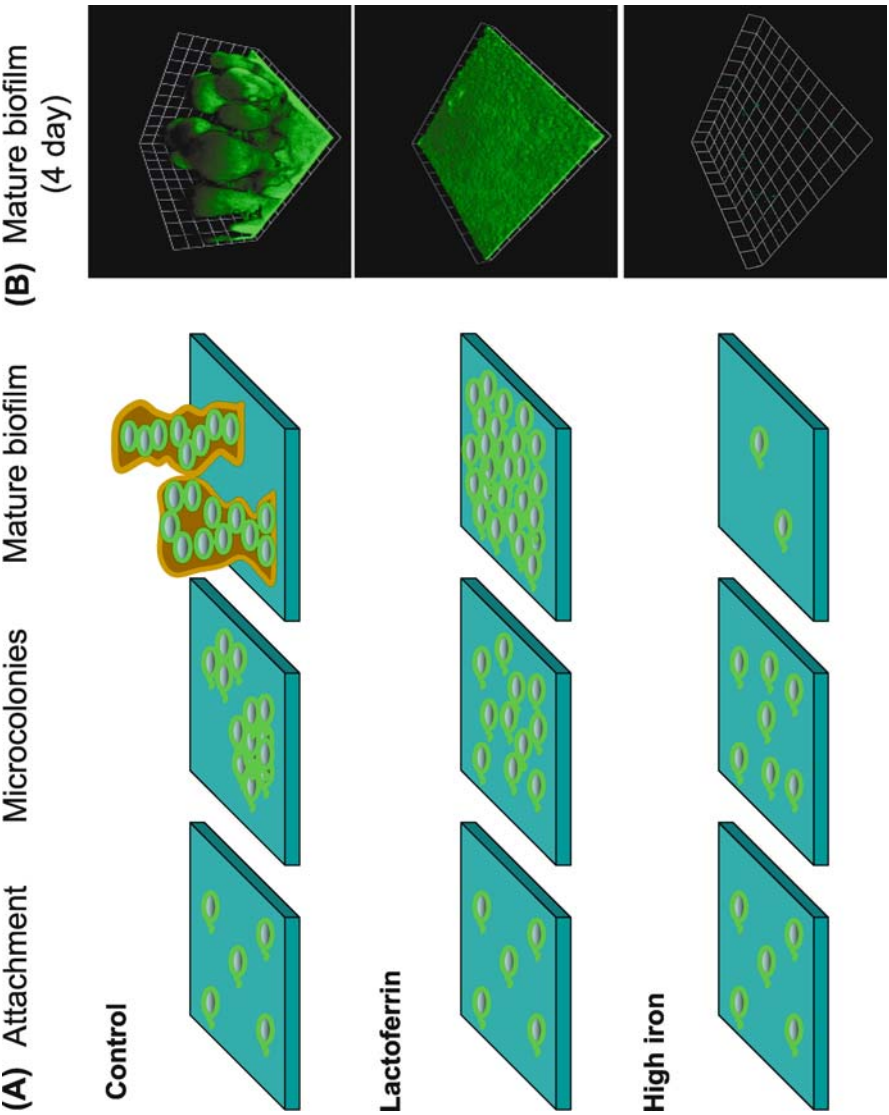

Fig. 1 Role of iron in *P. aeruginosa* biofilm development. **A** Schematic representation of the effects lactoferrin and high iron have on development of *P. aeruginosa* biofilms. Normally, bacteria attach, multiply, and develop into microcolonies that mature into structured biofilms. In the presence of subinhibitory concentrations of lactoferrin, the cells show increased surface motility; they attach and multiply, but daughter cells move away from the point of replication and thus do not form microcolonies or structured biofilms. Later, a thin, flat cell layer develops on the surface. In the presence of high iron, cells attach normally and multiply but do not remain attached to the glass surface, and biofilms do not form. **B** Effects of lactoferrin and high iron on *P. aeruginosa* biofilm development. GFP-labeled *P. aeruginosa* was grown for 4 days in continuous culture flow cells. The growth medium was 1% TSB (control) with subinhibitory concentrations of lactoferrin (20 μg/ml) or $FeCl_3$ (200 μM). Reconstructed three-dimensional images are presented; squares are 23 μm/side

This behavior was maintained as cells continued to divide and led to the formation of microcolonies. In the presence of lactoferrin, however, daughter cells used surface-associated motility, presumably twitching motility, to move away from the point of division. This constant movement on the surface interfered with the formation of sessile structures. The authors hypothesized that by chelating iron, lactoferrin induced twitching motility, which caused the cells to wander across the surface and thus disrupted their ability to settle and form structured communities. As support for this hypothesis, Singh et al. (2002) showed, by using a standard assay for twitching motility, that low iron induced *P. aeruginosa* twitching. Furthermore, they studied a twitching mutant in the flow-cell. Although not exactly resembling the wild-type biofilms in the absence of lactoferrin, the mutant was able to form microcolonies and structured biofilms even in the presence of lactoferrin.

The importance of iron for bacterial growth has been known for many years. The study by Singh et al. provided evidence that even when there is sufficient iron for planktonic growth, this element serves as a signal for biofilm development. In a recent paper, Berlutti et al. (2005) provided further evidence for the role of iron in cell aggregation and biofilm formation. They reported that in the liquid phase, iron limitation (1 μM) induced motility and transition to the planktonic mode of growth, while high iron concentrations (100 μM) facilitated cell aggregation and biofilm formation.

To further investigate the role of iron in biofilm development, we utilized a genetic approach and systematically screened mutants defective in iron acquisition and regulation for biofilm formation in the presence and absence of lactoferrin (Banin et al. 2005). Initially we examined the role of the two endogenous siderophores (pyoverdine and pyochelin) in biofilm formation. While biofilms formed by a pyochelin mutant were indistinguishable from the wild type, a pyoverdine mutant formed flat biofilms even in the absence of lactoferrin. Complementing the mutations or adding a conditioned medium containing pyoverdine restored normal biofilm development to the pyoverdine mutant. Because pyoverdine serves as an iron chelator and a signaling molecule (Lamont et al. 2002), we wanted to distinguish between these activities. We reasoned that if the activity is mediated through iron uptake, then the addition of iron should complement the mutation; however, if the activity is due to signaling, then the addition of iron would not influence biofilm development in a pyoverdine mutant. The addition of 50 μM iron was sufficient to restore biofilm formation in a pyoverdine mutant. This result suggested that the pyoverdine iron uptake activity, and not the signaling activity, is important for biofilm development. We also asked whether additional heterologous siderophores can restore biofilm formation in a pyoverdine mutant. We demonstrated that ferric citrate and ferrioxamine can both be utilized by *P. aeruginosa* pyoverdine mutant and that the pyoverdine mutant formed normal biofilms when these iron chelators were added to the culture medium. A *P. aeruginosa* genome sequence analysis revealed homologs of the ferric citrate and ferrioxamine uptake systems, and we showed that these systems are involved in the uptake of citrate or ferrioxamine chelated iron.

Our genetic analysis (Banin et al. 2005) indicated that *P. aeruginosa* requires an active iron uptake system to support biofilm development in vitro. It can utilize its endogenous siderophores pyoverdine (at low iron concentrations) and pyochelin (at high iron concentrations), or it can utilize other chelators for which it has an uptake system. Furthermore, by systemically screening mutants with defects in putative iron starvation ECF-sigma factors, we concluded that these systems are most likely not directly involved in signaling of biofilm development, but rather facilitate iron uptake of heterologous iron sources. Based on these results, we speculated that internal iron concentration is critical for normal biofilm development. Consistent with this hypothesis, mutants with abnormally functional Fur organized into structured biofilms under low iron, a condition in which the parent formed flat biofilms. The two known Fur-controlled small regulatory RNAs (PrrF1 and F2; Wilderman et al. 2004) did not appear to mediate iron control of biofilm development.

Besides being an essential nutrient for growth and a signal for biofilm development, iron stabilizes the extracellular polymeric matrix of *P. aeruginosa* biofilms (Chen and Stewart 2002). The viscosity of a mixed *P. aeruginosa–Klebsiella pneumoniae* biofilm suspension is affected by various cations. The addition of iron salts significantly increases biofilm viscosity. Electrostatic interactions contribute to biofilm cohesion, and iron cations are potent cross-linkers of the biofilm matrix. In support of these findings, we have demonstrated that chelation of iron, mediated by the addition of ethylenediamine tetraacetic acid (50 mM), initiated the detachment of cells from mature *P. aeruginosa* biofilms (Banin et al. 2006). Taken together, these results demonstrate that iron is important both as a signal for biofilm development and as a stabilizing cation of the biofilm matrix.

3.2 High Iron

The role of iron in biofilm development became even more intriguing with the work of Musk et al. (2005). Using a high throughput biofilm screen, the authors tested a library of over 4500 compounds for nonantibiotic biofilm inhibitors against *P. aeruginosa* PA14. One of the compounds that inhibited biofilm formation was ferric ammonium citrate. Neither ammonium nor citrate ion was responsible for this activity. Conversely, when other iron salts (ferric chloride, ferric sulfate, and ferrous sulfate) were examined, they showed biofilm inhibition similar to that of ammonium ferric citrate inhibition. The biofilm inhibition was significant at iron concentrations >100 μM, but this iron level did not affect *P. aeruginosa* growth. The authors utilized a flow-cell system to further characterize the inhibitory effect of iron on biofilm formation. Growing *P. aeruginosa* in flow cells in the presence of 200 μM ferric ammonium citrate completely inhibited biofilm formation, while in the absence of added iron, the cells formed robust structured biofilms (Fig. 1). The inhibition of biofilm formation was not due to reduced adhesion of cells to the surface, and initial biofilm formation at 10 h was similar under both conditions. Rather,

iron seemed to disrupt the later stages of biofilm development, leaving very few cells adhering to the surface by 48 h. Fourteen of the 17 clinical isolates that could form biofilms (82%) displayed >40% inhibition in biofilm formation when grown in the presence of 250 μM ferric ammonium citrate, and 41% had >60% inhibition. Interestingly, three of the strains were resistant to the treatment but were not further characterized.

Exposing mature biofilms to ferric ammonium citrate (200 μM) caused biofilms to disperse, resulting in complete clearing of the biofilms after 5 days (Musk et al. 2005). Thus, excess iron not only blocked biofilm formation but also disrupted existing biofilms. It is important to note that the addition of nutrients (such as carbon sources) to existing biofilms has been shown to induce biofilm dispersal, and this could be a more general response to nutrient excess rather than a specific response to iron (Sauer et al. 2004). The work by Musk et al. suggests that in vitro there is actually an optimal range of iron for biofilm growth and maintenance: approximately 1–100 μM) (Musk et al. 2005). When the iron concentration is too low (<1 μM) or too high (>100 μM), cells will abort normal biofilm development. It remains unclear by which mechanism high iron inhibits biofilm development and triggers dispersal.

4 Iron and Biofilm Formation In Vivo

The studies demonstrating an involvement of iron (both high and low) in biofilm development and persistence were carried out in vitro using biofilms grown on abiotic surfaces. An important question asks how relevant these findings are to the situation in vivo. The literature contains several interesting observations regarding the iron content in the CF lung that may shed light on the role iron plays in the ability to form biofilms in vivo.

4.1 Lactoferrin Activity in the Cystic Fibrosis Lung

An important piece of evidence supporting the in vitro findings comes from studies evaluating the activity of lactoferrin. Britigan et al. (1993) initially reported that lactoferrin underwent proteolytic cleavage in the lungs of CF patients, having detected lactoferrin cleavage products in bronchoalveolar lavage (BAL) samples from patients with CF. Such cleavage products were not detected in BAL samples from healthy controls. The authors also demonstrated partial cleavage of lactoferrin when lactoferrin was incubated with high concentrations of *Pseudomonas* elastase (an alkaline protease) and neutrophil elastase for prolonged periods. The authors speculated that this in vivo cleavage of lactoferrin and transferrin by neutrophil- and/or bacterial-derived proteases may have an important role in the acquisition of iron in the lungs by *P. aeruginosa*. In a more recent study, Jesaitis et al. (2003), investigating neutrophil interactions with *Pseudomonas* biofilms, postulated that a signif-

icant fraction of lactoferrin released during neutrophil degranulation was probably degraded by neutrophil-derived proteases. Rogan et al. (2004) demonstrated that cathepsins (lysosomal proteases) in CF secretions are responsible for complete and rapid cleavage of lactoferrin and that this cleavage results in the loss of lactoferrin's microbicidal and antibiofilm activity. Furthermore, lactoferrin levels and activity in sputum obtained from *P. aeruginosa*-infected and noninfected CF patients were compared. *P. aeruginosa*-positive sputum showed significantly higher cathepsin activity and lower levels of lactoferrin than *P. aeruginosa*-negative sputum. There was also reduced antimicrobial and antibiofilm lactoferrin activity in *P. aeruginosa*-positive compared with *P. aeruginosa*-negative sputum samples. Taken together, these studies suggest that lactoferrin in the CF lung is highly susceptible to cleavage and has substantial lower antimicrobial and antibiofilm activity compared with lactoferrin in healthy individuals.

4.2 Iron in the Cystic Fibrosis Lung

In light of the lactoferrin results, what is the iron content in the CF lung? It is estimated that the levels of free iron in human secretions are maintained at extremely low concentrations ($>10^{-18}$ M), which limits the growth of invading pathogens. Apparently, a high percentage of CF patients suffer from iron deficiency, which is attributed to a combination of factors such as poor dietary intake, inflammation, and gastrointestinal problems (Ehrhardt et al. 1987; Pond et al. 1996). In this condition the patient has a much lower iron pool than a healthy individual, and this implies that an invading bacterial pathogen might encounter even lower iron concentrations. However, work examining the iron content in the lungs suggests that despite the iron deficiency (which is measured as the amount of iron in the serum), sputum from CF patients is actually replete with iron. Stites et al. (1998, 1999) have examined the iron and ferritin content in the sputum of CF patients, and their results demonstrate that the concentrations of extracellular iron and ferritin are substantially higher in CF patients compared with healthy individuals. In addition, Reid et al. (2002) examined the correlation between iron deficiency, lung disease, and *P. aeruginosa* chronic infection. They demonstrated that iron and ferritin levels in the sputum of CF patients are much higher than in normal controls. These studies imply that the iron content in the lungs of CF patients is higher, which is advantageous for biofilm formation. However, we note that the confounding factor in these investigations is that the patients were already colonized with bacteria, which probably changed the environmental iron concentration.

4.3 P. aeruginosa Response to Iron In Vivo

It is well known that invading pathogens are in intense competition for iron with the host. However, iron content in the CF lungs might be substantially different from

that in healthy individuals, providing favorable conditions for infection and biofilm formation. Therefore, evaluating the iron response of *P. aeruginosa* in the lungs may serve as a marker for the type of environment the bacteria actually face. Haas et al. (1991) initially reported the identification of pyoverdine in sputum isolated from CF patients. This led to the conclusion that *P. aeruginosa* in the lungs most likely experiences a low iron environment and in turn secretes siderophores to scavenge iron. Wang et al. (1996) used an in vivo selection system (IVET) to identify *P. aeruginosa* genes that are specifically induced by respiratory mucus derived from CF patients. One of the genes they identified was FptA (the receptor for pyochelin); the induction was suppressed by the addition of exogenous iron, further demonstrating that the mucus is iron depleted.

It is now recognized that *P. aeruginosa* isolates from CF patients appear to originate from the environment but adapt to the milieu of the patient's airway. Beckmann et al. (2005) attempted to characterize the antigen response in early-infected CF patients during their first year of infection. By screening serum from these patients against a phage display peptide library, the authors were able to identify over 150 reactive clones. When these were sequenced and compared with the *P. aeruginosa* published genome, 76 genes encoding outer membrane and secreted proteins were identified. The majority of these were proteins involved in small-molecule transport, membrane structural proteins, and secreted factors. Interestingly, several of the genes identified by this technique were involved in iron acquisition (e.g., FptA and several siderophore receptor proteins). Furthermore, the transcriptome responses of *P. aeruginosa* exposed to differentiated human epithelia has revealed a significant overlap with the iron-responsive regulon (Chugani and Greenberg 2007). Of the set of 97 genes identified in a previous study as induced upon growth in iron-deficient conditions, 80% were induced when *P. aeruginosa* was exposed to epithelia. Taken together, these studies suggest that the bacteria are challenged by the deficiency of available iron in the epithelial milieu during the initial phase of infection.

If one continues to follow the adaptation of *P. aeruginosa* as a marker for the iron content in the airways, there is evidence that this condition may change as the infection progresses. A study examining genomic variation in *P. aeruginosa* strains isolated from children with CF (6 months to 8 years of age) revealed that several strains had a deletion in the pyoverdine locus, which eliminated biosynthesis and uptake of pyoverdine (Ernst et al. 2003). It actually appears that the pyoverdine locus is highly divergent and that some of the *pvd* genes may be under positive selective pressure (Smith et al. 2005). De Vos et al. have also reported that pyoverdine synthesis mutants seemed to accumulate as a function of the lung colonization time (De Vos et al. 2001). Although this is highly speculative, the accumulation of mutants that lose their major and most powerful siderophore (i.e., pyoverdine) may imply that the conditions in the lungs (as the disease progresses) change during colonization from iron-limiting to iron-sufficient.

5 Iron as a Signal for Biofilm Formation in Other Bacteria

Is iron involved in signaling biofilm development of other bacterial species? And if so, what is the mechanism? We found several reports in the literature that have addressed these questions (summarized in Table 1). O'Toole and Kolter (1998b) reported that, similar to our observation of *P. aeruginosa*, the addition of iron could restore biofilm development by several *Pseudomonas fluorescens* attachment mutants. *Vibrio cholerae* also requires iron for normal biofilm development. A wild-type *V. cholera* strain formed reduced biofilms in an iron-deficient medium compared with iron-replete conditions (Mey et al. 2005). This iron response is mediated in part by Fur through the sRNA *ryhB*. A *ryhB* mutant showed reduced chemotaxis in low iron and was unable to form wild-type biofilms. The biofilm defect was suppressed by the addition of iron (Mey et al. 2005).

Interestingly, we also found several reports on the negative regulation of iron on biofilm formation. In the gram-negative human pathogen *Acinetobacter baumannii*, biofilm formation is significantly enhanced under low iron conditions (Tomaras et al. 2003). The authors characterized a novel chaperone/usher pilus system that is involved in biofilm development and identified a potential Fur box upstream of the first gene in the operon. The authors speculated that this Fur-mediated pilus synthesis results in reduced biofilm formation under high iron conditions. Two gram-positive bacteria (*S. mutans* and *S. aureus*) also demonstrated improved biofilm formation under iron-depleted conditions. Berlutti et al. (2004) reported that iron-deprived saliva (<0.1 μM Fe^{3+}) increases *S. mutans* aggregation and biofilm formation, whereas iron-loaded saliva (>1 μM Fe^{3+}) inhibits both phenomena. These results suggest that aggregation and biofilm formation are negatively controlled by iron. The authors hypothesized that lactoferrin that is readily found in saliva may mediate this response. In agreement with this hypothesis, subinhibitory concentrations of lactoferrin stimulated biofilm formation, while iron-saturated lactoferrin decreased aggregation and biofilm formation (Berlutti et al. 2004). In *Staphylococcus aureus*, iron depletion was shown to promote biofilm formation, and this response was partially regulated by Fur (Johnson et al. 2005).

Table 1 Iron and biofilm development in diverse bacteria

Strain	Biofilm development in low iron	Motile organism	Mode of regulation	Refs.
P. aeruginosa	Reduced	Yes	Fur	Banin et al. 2005; Singh et al. 2002
P. fluorescens	Reduced	Yes	Unknown	O'Toole and Kolter 1998b
V. cholera	Reduced	Yes	Fur, RyhB	Mey et al. 2005
A. baumannii	Increased	No	Fur (?)	Tomaras et al. 2003
S. mutans	Increased	No	Unknown	Berlutti et al. 2004
S. aureus	Increased	No	Fur (partially)	Johnson et al. 2005

One common theme that arises when comparing the role iron plays in mediating biofilm development in all these bacterial species is motility (see Table 1). The three bacterial species that showed reduced biofilm formation under low iron conditions are motile bacteria (*P. aeruginosa*, *P. fluorescens*, and *V. cholera*). All of the nonmotile bacteria (*A. baumannii*, *S. mutans*, and *S. aureus*) had induced biofilm formation under iron-depleted conditions. Motility is crucial to biofilm development in several motile species (O'Toole and Kolter 1998a; O'Toole et al. 2000). Furthermore, in the case of *P. aeruginosa*, induction of surface motility has provided an explanation for impaired biofilm formation in the presence of lactoferrin (Singh et al. 2002; Singh 2004). An important question is whether surface motility is directly controlled by iron directly or whether iron controls cell–cell adhesion, which in turn affects motility and biofilm formation. Further work will be needed to characterize the role motility plays in mediating the dependence of biofilm development on iron and how it is regulated.

6 Iron Metabolism as an Antibiofilm Target

The absolute requirement of iron for growth in most bacteria makes iron an appealing target for antimicrobial therapy. An initial concept was to replace Fe^{3+} with a nonmetabolizable metal ion such as scandium or indium in an effort to block iron uptake by competitive inhibition of the transport proteins (Rogers et al. 1982). Although initial results were encouraging for Sc^{3+} treatment of *Escherichia coli* and *Klebsiellla pneumoniae* mouse infections (Rogers et al. 1980), Sc^{3+} proved toxic to the animals (Plaha and Rogers 1983). A similar approach was taken in *Mycobacterium tuberculosis* using molybdenum, cobalt, and scandium, but with no major effect (Barclay and Ratledge 1986). A promising development came with the use of gallium-portoporphyrin, which had antimicrobial activity against *Yersinia enterocolitica*, methicillin-resistant *S. aureus*, and *Mycobacterium smegmatis* (Stojiljkovic et al. 1999). The gallium-porphyrin complex enters bacteria via heme transport systems and was assumed to interfere with respiration, generating reactive oxygen radicals that presumably killed the cells (Stojiljkovic et al. 1999). The gallium complexes had no toxic effect on human fibroblasts or other animal cell lines, further highlighting the potential for clinical use.

Another strategy to target bacterial homeostasis was the use of siderophore-antibiotic conjugates. This "Trojan horse" approach is based on molecules found in nature, such as albomycins, which contain an iron-binding domain (similar to ferrichrome) and a toxic molecule. Several microorganisms can introduce albomycin through the ferrichrome uptake system. Once internalized, the toxic molecule is cleaved and damages the cell (Benz et al. 1982). Imitating this strategy, several groups have chemically synthesized siderophore-antibiotic complexes and tested them for their antimicrobial activity. The most common approach was to conjugate beta-lactam antibiotics with a siderophore moiety (Ghosh et al. 1996; Ghosh and Miller 1995). These complexes show excellent anti-*Pseudomonas* activity in vitro

and in several animal infection models (Basker et al. 1984, 1989; Budzikiewicz 2001). The emerging data on the role of iron as a *P. aeruginosa* biofilm signal suggests that these Trojan-horse approaches might be particularly well suited for treating chronic *P. aeruginosa* infections.

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