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Iron acquisition by *Pseudomonas aeruginosa* in the lungs of patients with cystic fibrosis

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Abstract The bacterium *Pseudomonas aeruginosa* is commonly isolated from the general environment and also infects the lungs of patients with cystic fibrosis (CF). Iron in mammals is not freely available to infecting pathogens although significant amounts of extracellular iron are available in the sputum that occurs in the lungs of CF patients. *P. aeruginosa* has a large number of systems to acquire this essential nutrient and many of these systems have been characterised in the laboratory. However, which iron acquisition systems are active in CF is not well understood. Here we review recent research that sheds light on how *P. aeruginosa* obtains iron in the lungs of CF patients.

Keywords Pseudomonas aeruginosa · Chronic infection · Cystic fibrosis · Iron acquisition · Siderophore · Pyoverdine · Pyochelin · Infectious disease

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Cystic fibrosis and *Pseudomonas aeruginosa*

The genetic condition cystic fibrosis (CF) is the most common lethal disease amongst the Caucasian population, occurring at an incidence of approximately 1 in 2,500 live births. Individuals with CF experience a range of clinical manifestations but chronic lung disease is well accepted as the leading cause of morbidity and mortality amongst these patients (Davis 2006; Davies et al. 2007). The morbidity and mortality associated with cystic fibrosis is predominantly related to an increased and relentless rate of lung function decline, interspersed by sporadic declines termed pulmonary exacerbations that serve to accelerate the process. Exacerbations are characterised by increases in coughing, sputum volume and breathlessness and may cause fever and weight loss.

The lungs of CF patients contain thick, viscous mucus that lines the airways and is difficult to clear. The resulting environment is conducive to bacterial infection with *Pseudomonas aeruginosa* being the most common isolate, followed by *Staphylococcus aureus* and *Hemophilus influenzae* (Ratjen and Doring 2003). *Burkholderia cepacia* and related species are also important pathogens in CF (Mahenthiralingam et al. 2005; Chiarini et al. 2006). The microflora in CF is very complex, with multiple species often present (Harrison 2007). A recent study identified over 100 different bacterial species in bronchoalveolar lavage fluid from a total of 28 patients (Harris et al. 2007). The population of organisms present in each patient

differed considerably. There is significant variation between the strains and species present in the upper and lower airways, and even between different lobes of the lung (Smith et al. 1998; Nixon et al. 2001).

P. aeruginosa has been a major focus of research attention because it is present in the lungs of the majority of CF patients, often in large numbers (over 10^8 cfu per ml of sputum) (e.g. Aaron et al. 2004; Reid et al. 2007) and infection with *P. aeruginosa* is correlated with a progressive and relentless decline in patient health (Burns et al. 2001; Emerson et al. 2002; Konstan et al. 2007). *P. aeruginosa* is a saprophytic bacterium that can be isolated from a range of environmental niches (Palleroni 1981). In CF, infection with *P. aeruginosa* persists despite a robust humoral and cellular chronic immune response and extensive antibiotic treatment. Strains of *P. aeruginosa* that colonise CF patients are genetically as diverse as those found in the general environment (Wiehlmann et al. 2007), suggesting that all strains of *P. aeruginosa* have the ability to infect CF patients, although there is also evidence for infectious clones ("epidemic strains") that have an increased ability to cause infection (reviewed in Govan et al. 2007). Once infection is established the same strain usually remains present in patients although it is likely to undergo genetic adaptation to the CF lung environment (Smith et al. 2006). Some instances of superinfection by other strains have been reported (McCallum et al. 2001).

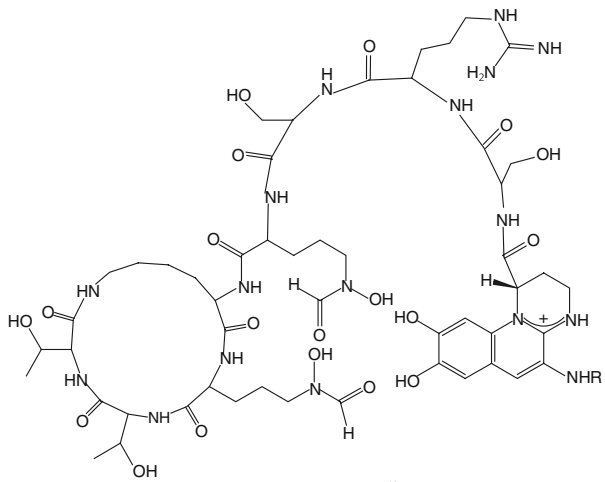
There is considerable evidence that in CF *P. aeruginosa* exists primarily in biofilms rather than in a free-floating planktonic state (Lam et al. 1980; Singh et al. 2000; Yang et al. 2008). Biofilms are highly structured bacterial communities that are encased in a biopolymer matrix and gene expression in biofilms is very different from that of planktonic bacteria (Costerton et al. 1999; Stoodley et al. 2002; Hall-Stoodley et al. 2004). Biofilms confer significant antibiotic resistance and are difficult to eradicate (VanDevanter and Van Dalfsen 2005). Oxygen availability in biofilms in the CF lung may be low (Worlitzsch et al. 2002; Yoon et al. 2002) which would be expected to significantly affect the physiology of the bacteria.

Iron and infection

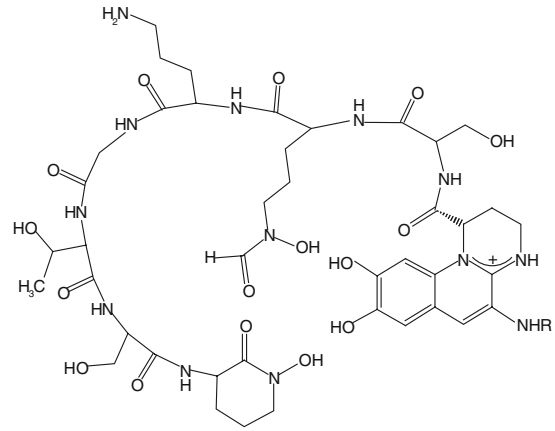
Bacteria require iron as a cofactor for numerous enzymes essential for metabolism. Free iron in biological solutions at pH 7.0 is present as oxidised Fe^{3+} and is present at concentrations of 10^{-9} M or less which is too low to be sufficient for bacterial growth (Bullen et al. 1978; Ratledge and Dover 2000). In mammals, most iron is bound by or incorporated into proteins (primarily ferritin, transferrin, lactoferrin and hemoglobin) with high affinity (Kd $\sim$ 10^{20} for the extracellular proteins transferrin and lactoferrin). This has been termed "iron withholding" as it reduces the bioavailability of iron to infecting bacteria and it is an important component of innate immunity against bacterial infection (Weinberg 1984; Ratledge and Dover 2000). Its importance is emphasized by experiments with very many species showing that addition of iron in models of infection increases bacterial pathogenicity (reviewed in Ratledge and Dover 2000); for example, addition of iron reduced the LD50 of a strain of *P. aeruginosa* by up to 1,000-fold in a mouse model of infection (Forsberg and Bullen 1972). Iron saturation of host proteins is reduced during infection as part of the systemic inflammatory response, further lowering the amount of iron available to infecting bacteria (Weinberg 1984).

Many bacteria acquire iron through the secretion of siderophores, which are iron-scavenging molecules with high affinities for Fe^{3+} ions (typically with formation constants in the range 10^{22} to 10^{35}, though siderophores with both higher and lower affinities are known; Matzanke 2005). There are numerous examples of siderophores made by pathogenic bacteria that have been shown to be required for normal infection (reviewed in Ratledge and Dover 2000; Bullen et al. 2005). *P. aeruginosa* makes two kinds of well-characterised siderophores, pyoverdines and pyochelin (Fig. 1). Pyoverdines have iron formation constants between 10^{24} and 10^{27} at pH 7.0 (Budzikiewicz 2004). Strains of *P. aeruginosa* make one of three different pyoverdines designated Type I, II and III (Meyer et al. 1997). Each pyoverdine has a specific receptor for its uptake (FpvAI, FpvAII or FpvAIII) (Poole et al. 1993; de Chial et al. 2003) and each strain only expresses the FpvA receptor for the type of pyoverdine that it produces. A second receptor for uptake of Type I pyoverdine (FpvB) enables Type II and Type III strains to use Type I pyoverdine (Ghysels et al. 2004).

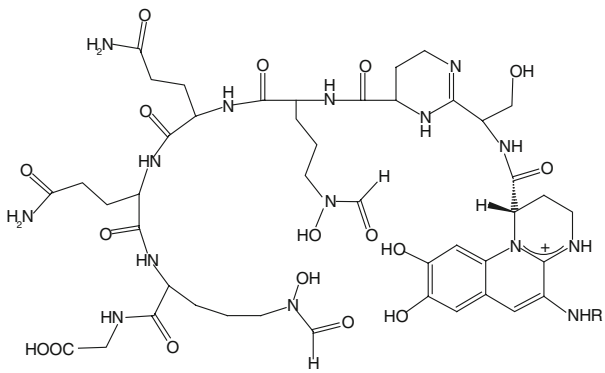
Pyoverdine synthesis and ferripyoverdine uptake is best characterised for *P. aeruginosa* strain PAO1 that



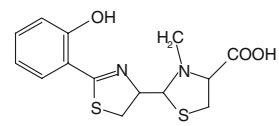
Type I Pyoverdine



Type II Pyoverdine



Type III Pyoverdine



Pyochelin

Fig. 1 Siderophores synthesised by *Pseudomonas aeruginosa*. Each pyoverdine is composed of a fluorescent dihydroxyquinoline chromophore attached to an acyl chain (R) and a Type-specific peptide. Fe^{3+} is bound by the catecholate and hydroxamate groups (Teintze et al. 1981; Tappe et al. 1993; Wasielewski et al. 2008). Pyochelin is thought to bind iron in a 1:1 ratio (Schlegel et al. 2004; Tseng et al. 2006) but the structure of the ferripyochelin complex has not been determined

makes Type I pyoverdine (reviewed in Schalk 2007; Visca et al. 2007). Synthesis requires the coordinated action of at least 15 enzymes located in the cytoplasm and the periplasm of the bacteria. Uptake of ferripyoverdine is primarily mediated by an outer membrane protein FpvA, acting in conjunction with the TonB energy-transducing protein to import ferripyoverdine into the periplasm. Synthesis of pyochelin by *P. aeruginosa* has also been well studied (Crosa and Walsh 2002) with uptake of ferripyochelin being via the cell surface receptor FptA (Ankenbauer and Quan 1994).

Pyoverdine is generally considered the primary siderophore for *P. aeruginosa.* It has a higher affinity for iron than pyochelin ($2.5 \times 10^5\ M^{-1}$; Cox and Graham 1979) and is more effective than pyochelin at releasing iron from transferrin (Sriyosachati and Cox 1986). Pyoverdine was necessary for infection in an animal model of severe burn wounds (Meyer et al. 1996) and was of more importance than pyochelin for infection in immunosuppressed mice (Takase et al. 2000). However pyochelin also contributed to bacterial virulence in this study, mirroring earlier findings (Cox 1982).

Pyoverdine-mediated iron transport is important for biofilm development (Banin et al. 2005; Patriquin et al. 2008). Mutant strains of *P. aeruginosa* that do not synthesize pyoverdine made defective

biofilms under conditions of iron starvation and addition of pyoverdine restored normal biofilm formation. Pyochelin was not sufficient to enable complete biofilm development in the absence of pyoverdine, but addition of heterologous siderophores desferrioxamine and ferric citrate to the pyoverdine-deficient mutant restored biofilm phenotype to that of wild-type bacteria (Banin et al. 2005). Iron starvation is associated with changes in quorum sensing and twitching motility, both of which are important factors in biofilm development (Patriquin et al. 2008). These data, demonstrating the need for siderophore-mediated iron transport in normal biofilm development, are consistent with earlier findings that iron sequestration by lactoferrin inhibits *P. aeruginosa* biofilm development and affects twitching motility that is essential for biofilm development (Singh et al. 2002). Conversely, high amounts of iron also inhibit biofilm formation (Musk et al. 2005; Yang et al. 2007; Musk and Hergenrother 2008).

P. aeruginosa has the capacity to use a wide range of siderophores synthesized by other organisms (reviewed in Cornelis and Matthijs 2002; Poole and McKay 2003) and has at least two systems for uptake of heme (Ochsner et al. 2000). Genome analysis indicates that *P. aeruginosa* also has the capacity to acquire Fe^{2+} ions through a FeoAB transport system (reviewed in Cartron et al. 2006) and through an EfeU-type system (Grosse et al. 2006; Cao et al. 2007) but this has not been examined experimentally.

How does *P. aeruginosa* obtain iron in CF lungs?

Within the healthy airway, iron is predominantly found in iron binding proteins such as transferrin, lactoferrin and ferritin, whilst only a minute amount is present in a freely available form (Mateos et al. 1998). However, sputum from CF patients contains significant amounts of iron (average of four studies of 1.2 μg/ml; Stites et al. 1998, 1999; Reid et al. 2004, 2007). Some of this is in the form of ferritin (average of 1.7 μg/ml in the four studies, significantly higher than in control samples). Elevated iron levels were positively correlated with *P. aeruginosa* bacterial load in stable infected patients although the relationship in exacerbating patients was less clear (Reid et al. 2007). These data show that CF airways are not fully iron-deplete environments and suggest that iron is present in sufficient quantities to support bacterial growth. They are consistent with findings that lung epithelial cells with a mutation in the CF gene accumulate and release significant amounts of extracellular iron when grown in culture, whereas cells with the functional gene do not (Moreau-Marquis et al. 2008).

As well as ferritin, the human airway contains lactoferrin and transferrin. Pyoverdine can acquire iron from both of these proteins (Sriyosachati and Cox 1986; Xiao and Kisaalita 1997). Furthermore, proteases that are secreted by *P. aeruginosa* can degrade lactoferrin and transferrin and increase the ability of pyoverdine to acquire iron from these molecules (Doring et al. 1988; Wolz et al. 1994). Transferrin and lactoferrin undergo proteolysis in CF (Britigan et al. 1993) providing a potential supply of iron to the bacteria.

Iron availability for *P. aeruginosa* in CF is complicated by the presence of other infecting organisms that may compete for available iron, but may also increase its bioavailability. Microbial inter-species iron transactions have been little studied but competition for iron may have a major influence on bacterial growth. In one in vitro study that is relevant to CF the presence of *S. aureus* caused reduced expression of iron uptake genes of *P. aeruginosa*, implying that *P. aeruginosa* was able to obtain iron from *S. aureus* (Mashburn et al. 2005). Conversely, co-culture of *P. aeruginosa* with *Burkholderia* spp. caused increased expression of iron-responsive genes in *P. aeruginosa*, including those involved in pyoverdine and pyochelin synthesis, because of iron sequestration by the *Burkholderia* siderophore ornibactin (Weaver and Kolter 2004). *Burkholderia* species from patients with CF also make pyochelin (Sokol 1986; Darling et al. 1998) that could deliver iron to *P. aeruginosa*. *B. cepacia* and *P. aeruginosa* can form mixed biofilms (Eberl and Tummler 2004), providing intimate interactions between the bacteria that have a high potential for competition for iron but also for exchange of siderophores and iron cross-feeding.

In light of the above we can ask the question, do pyoverdine and pyochelin contribute to iron acquisition by *P. aeruginosa* in CF? An initial approach to this question involved purification of pyoverdine from CF sputa (Haas et al. 1991a). Pyoverdine was

successfully purified from six out of twelve sputum samples, in amounts corresponding to a mean concentration of just under 1 μmol in the sputa. The majority of the pyoverdine (54–88%) was ferrated. Spectral analysis indicated that pyoverdine was present in the remaining six samples but the amounts were too low for purification. We have refined these methods and directly measured the amounts of pyoverdine in CF sputum and detected pyoverdine in sputum from 25 of 28 patients in amounts ranging from 0.7 to 51 μmol (I. Lamont, L. Martin and D. Reid, manuscript in preparation). The presence of pyoverdine in most CF sputa implies that is has a role in iron acquisition by the bacteria.

Six isolates of *P. aeruginosa* from CF patients were all able to make pyochelin (Haas et al. 1991b), but this compound has a much lower affinity for iron than pyoverdine, or the host iron-binding proteins lactoferrin and transferrin, so that its significance as an iron-scavenging agent during infection is not clear. So far as we are aware, pyochelin has not been identified in CF sputum.

A second approach to attempting to understand iron acquisition by *P. aeruginosa* in CF is to analyze the effects of sputum on gene expression. Microarray analysis showed that the presence of sputum in growth medium induced expression of a wide range of iron-acquisition genes including those involved in synthesis and uptake of pyoverdine and pyochelin and the *hasA* gene that enables heme uptake (Palmer et al. 2005). Microarray analysis has also been carried out using RNA extracted directly from sputum from a CF patient (Son et al. 2007). Bacteria in the sputum had increased expression of pyochelin synthesis genes but not pyoverdine synthesis genes, relative to bacteria grown in laboratory minimal medium.

Most characterised CF strains of *P. aeruginosa* make and use Type I or Type II pyoverdine (Meyer et al. 1997; De Vos et al. 2001) although an epidemic strain uses Type III pyoverdine (de Chial et al. 2003). However, in genotyping analysis of *P. aeruginosa* from CF the three FpvA receptor types occurred with similar frequencies (Wiehlmann et al. 2007). Intriguingly, some isolates from patients who had been chronically infected for a long time had lost the ability to make pyoverdine, although they retained the ability to take up ferripyoverdine (De Vos et al. 2001). A similar finding was made in a longitudinal study of chronic *P. aeruginosa* infection in a single patient (Smith et al. 2006). These bacteria may utilize pyoverdine made by other *P. aeruginosa* in CF or may acquire iron through a different uptake system. The concentrations of iron in some CF sputa are greater than 10 μM, which is sufficient to suppress pyoverdine-mediated transport in vitro (Meyer and Abdallah 1978). If *P. aeruginosa* is exposed to such concentrations for a prolonged period in CF, mutations in the pyoverdine system could occur without being biologically disadvantageous. Many strains of *P. aeruginosa* in CF have a high rate of mutation (Oliver et al. 2000) increasing the likelihood of mutations in genes that are not necessary for survival and growth in chronic infections that span many years.

P. aeruginosa may also acquire iron via siderophore-independent pathways in CF. Pulmonary exacerbations may lead to haemolysis resulting in the presence of heme in sputum. This would be a potential source of iron for *P. aeruginosa* via heme uptake pathways. In addition, under low oxygen conditions such as may be experienced by the bacteria in biofilms in CF iron may be in the ferrous (Fe^{2+}) form. This could be taken up by *P. aeruginosa* via the FeoABC (and EfeU) pathways. There are also suggestions that the airways in CF are abnormally acidified (Tate et al. 2002), potentially increasing the pool of Fe^{2+} as acidic conditions protect Fe^{2+} ions against oxidation.

Conclusions and future challenges

Siderophore-mediated iron uptake by *P. aeruginosa* in the laboratory is well understood and there is reasonable knowledge of other iron uptake pathways. The challenge is to relate knowledge gained in vitro to iron acquisition in CF. Recent research has shown that the amount of extracellular iron in CF sputum is much higher than in other biological fluids but the form of this iron needs to be determined before we can properly understand how it might be acquired by *P. aeruginosa*. The presence of pyoverdine in sputum from CF patients indicates that this siderophore plays a role in iron acquisition in CF but its absence from some samples, coupled to the occurrence of pyoverdine-deficient mutants in CF, show that it is not the only mechanism of iron uptake and indeed, other iron-acquisition pathways may play a dominant role in some patients. The iron acquisition pathways used

by *P. aeruginosa* in CF may be quite variable and different in individual patients, depending on the exact nature of the environment and the form(s) and amount of iron that is present. In addition to better understanding of the forms of iron present, it will be a major challenge to understand the complex interplay between *P. aeruginosa* and other bacteria and how this affects iron acquisition. How all of these factors interact to influence the biofilm mode of growth that is thought to occur in CF will require extensive study in vitro as well as in vivo. However, it is clear that iron acquisition is crucial to the survival and growth of *P. aeruginosa* in CF and a proper understanding of the mechanisms used holds the prospect of identifying new and effective ways of treating infections by this key pathogen.

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