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Iron acquisition in the cystic fibrosis lung and potential for novel therapeutic strategies

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Iron acquisition is vital to microbial survival and is implicated in the virulence of many of the pathogens that reside in the cystic fibrosis (CF) lung. The multifaceted nature of iron acquisition by both bacterial and fungal pathogens encompasses a range of conserved and species-specific mechanisms, including secretion of iron-binding siderophores, utilization of siderophores from other species, release of iron from host iron-binding proteins and haemoproteins, and ferrous iron uptake. Pathogens adapt and deploy specific systems depending on iron availability, bioavailability of the iron pool, stage of infection and presence of competing pathogens. Understanding the dynamics of pathogen iron acquisition has the potential to unveil new avenues for therapeutic intervention to treat both acute and chronic CF infections. Here, we examine the range of strategies utilized by the primary CF pathogens to acquire iron and discuss the different approaches to targeting iron acquisition systems as an antimicrobial strategy.

CYSTIC FIBROSIS (CF) AND THE LUNG MICROBIOME

CF is an autosomal genetic disorder and is the most common lethal genetic disease amongst Caucasians, with >30000 patients currently on the European Cystic Fibrosis Society registry and a projected increase of 50% in the number of CF patients in the European Union by 2025 (Burgel *et al.*, 2015). CF is caused by a defect in the CF transmembrane conductance regulator (CFTR) gene resulting in a decreased airway surface liquid and increased mucous viscosity in the CF lung, leading to impaired mucociliary clearance of micro-organisms (Boucher, 2007). Due to this impairment, ~90% of CF patients succumb to respiratory failure as a result of chronic bacterial infections (Lubamba *et al.*, 2012). *Pseudomonas aeruginosa* is the pathogen of highest prevalence and incidence in the CF lung with 50–60% of adult CF patients colonized, followed in prevalence by *Staphylococcus aureus* (LiPuma, 2010). The epidemiology of CF pathogens has changed in the last decade, and in a retrospective analysis of the data reported to the CF Foundation Patient Registry in the USA, the prevalence and incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) had significantly increased in CF patients (Salsgiver *et al.*, 2015). Although of much lower prevalence (<5%), *Burkholderia cepacia* complex (Bcc) organisms are particularly problematic in CF due to their transmissibility and in a subset of patients can lead to a fulminant pneumonia with a high mortality rate (Mahenthiralingam *et al.*, 2005). *Haemophilus influenzae* is associated with early stage CF with up to 30% of those under 5 years colonized, compared with <10% of adult CF patients (LiPuma, 2010). In addition to MRSA, other emerging pathogens in CF include *Achromobacter xyloxidans*, *Stenotrophomonas maltophilia*, non-tuberculous mycobacteria and *Mycobacterium avium* (Parkins & Floto, 2015; Salsgiver *et al.*, 2015). Also represented are the genera *Streptococcus*, *Prevotella*, *Rothia*, *Veillonella* and *Actinomyces* (Coburn *et al.*, 2015). Non-culture-based identification methods have increased the detection of fungi in CF, with *Aspergillus fumigatus* being the most frequently isolated (Sabino *et al.*, 2015) and a decline in lung function appears to be a risk factor for *Aspergillus fumigatus*, which in turn is associated with a further deterioration in chronically colonized patients (Noni *et al.*, 2015). *Candida albicans*, a frequent colonizer of the CF lung, is also associated with a significant decline in lung function in chronically colonized patients (Gileles-Hillel *et al.*, 2015). Despite the complexity of the microbiome of the CF lung, a recent study has shown that the microbial community is stable during periods of clinical stability. However, with the onset of clinical exacerbations, a decrease in the dominant taxa was observed in some patients (Carmody *et al.*, 2015). The advent of high-throughput detection methods to profile CF microbiota can facilitate the rapid identification of colonizing organisms and detect changes in microbial diversity as disease progresses

Abbreviations: ABC, ATP-binding cassette; Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; CFEM, common fungal extracellular membrane; CFTR, cystic fibrosis transmembrane conductance regulator; MRSA, meticillin-resistant *Staphylococcus aureus*; NRPS, non-ribosomal protein synthetase; TBDR, TonB-dependent receptor.

(Flight *et al.*, 2015), and molecular signatures of species associated with different levels lung function may have potential as predictive biomarkers for clinical deterioration in CF (Paganin *et al.*, 2015). The emerging data on changes in the bacterial community of the CF lung are the subject of a recent review by Caverly *et al.* (2015). As the complexity and dynamic nature of the CF microbiome are being increasingly characterized, microbiologists are continually challenged to understand the potential of these organisms to contribute to CF lung disease and to develop targeted strategies against the main perpetrators.

IRON AND CF

CF pathogens, similar to other pathogenic micro-organisms, require iron for many basic cellular functions and metabolic pathways. However, iron metabolism is tightly regulated to prevent iron toxicity and to limit iron availability to pathogens, as recently reviewed by Ganz & Nemeth (2015). The liver hormone hepcidin plays a key role in iron homeostasis by causing degradation of the iron transporter ferroportin, thereby blocking iron flow into the plasma from cellular stores and from dietary sources (Nemeth *et al.*, 2004b). Hepcidin is elevated in response to iron and also to inflammatory signals (Nemeth *et al.*, 2004a), and is downregulated when iron levels are diminished (Donovan *et al.*, 2005), thus playing a central role in iron homeostasis. Iron is recycled from senescent erythrocytes by macrophages and redistributed back to the bone marrow (Beaumont & Delaby, 2009). Coupled with maintaining iron homeostasis, the availability of iron for pathogens is tightly restricted. More than half of the total bodily iron is bound to haemoglobin in erythrocytes, approximately a quarter of bodily iron is stored within cells bound to ferritin and transferrin chaperones iron in plasma (Ganz & Nemeth, 2015). These proteins, together with other iron-sequestering proteins including lipocalins that sequester iron from bacterial siderophores (Goetz *et al.*, 2002; Fluckinger *et al.*, 2004), play a significant role in iron-targeted nutritional immunity. The battle between host iron sequestration and *Staphylococcus aureus* iron acquisition typifies these complex relationships, and is detailed in a review by Haley & Skaar (2012).

Host iron metabolism is, however, altered during infection, with increased levels of iron-scavenging activity by both the host and pathogen (Parrow *et al.*, 2013). An increase in hepcidin in response to inflammatory mediators, including IL-6, results in hypoferraemia, commonly associated with inflammation (Nemeth *et al.*, 2004a) and with a prevalence of 62–72% in CF patients (Pond *et al.*, 1996; Reid *et al.*, 2002). In a recent study of CF pulmonary exacerbation, patients following treatment with intravenous antibiotics had lower hepcidin and IL-6 levels in serum, increased serum iron, and a trend towards lower sputum iron levels – further evidence of the impact of infection and inflammation on iron metabolism (Gifford *et al.*, 2012). Not surprisingly, given the inflammatory environment of the CF lung, elevated levels of iron and ferritin have been detected in CF sputum by a number of studies (Stites *et al.*, 1998, 1999; Reid *et al.*, 2004, 2007; Gray *et al.*, 2010). More recently in a comprehensive study of iron in CF, Ghio *et al.* (2013) demonstrated increased levels of iron and iron-related proteins in bronchoalveolar lavage fluids from CF children, in macrophages of explanted CF lungs and in lung tissue from CF patients – clear evidence of altered iron homeostasis and of iron accumulation in the CF airways (Ghio *et al.*, 2013). Consistent with these findings, Moreau-Marquis *et al.* (2008) demonstrated that airway cells expressing ΔF508-CFTR released more extracellular iron than cells rescued with WT-CFTR (Moreau-Marquis *et al.*, 2008). Furthermore, iron and ferritin levels are positively correlated with c.f.u. of *P. aeruginosa* and remain elevated after antibiotic treatment of *P. aeruginosa* infection (Reid *et al.*, 2007). *Aspergillus fumigatus* co-cultured with macrophages downregulates expression of two cellular iron importers and the iron exporter ferroportin, which, together with an increase in iron retention and ferritin synthesis by the exposed macrophages, provides further evidence of the impact of infection on host iron homeostasis (Seifert *et al.*, 2008). The majority of the iron is associated with ferritin and in the ferric (Fe^{3+}) form with limited bioavailability; however, CF pathogens have a range of mechanisms by which they can form usable ferrous (Fe^{2+}) iron and thereby thrive in this niche. The acidic environment of the CF lung (Tate *et al.*, 2002) may limit Fe^{2+} oxidation, further enhancing ferrous iron levels. Recently, Hunter *et al.* (2013) determined that the relative balance of ferric and ferrous iron in the CF lung changes as infections progress and over time ferrous iron dominates (Hunter *et al.*, 2013). Increased airway iron in CF therefore facilitates the colonization by pathogens having the capacity to exploit this resource. There is a variety of evidence implicating iron acquisition in microbial pathogenesis. Over a decade ago, Berlutti *et al.* (2005) demonstrated that iron availability impacts on biofilm formation, adhesion and invasion of two important CF pathogens, *P. aeruginosa* and *Burkholderia cenocepacia*. More recently, Wiens *et al.* (2014) demonstrated that alginate production, mucoid phenotype and biofilm formation by *P. aeruginosa* are iron regulated.

In this review we examine what is currently known about iron acquisition by the main CF pathogens and focus on four key mechanisms: production of iron-binding siderophores, haem uptake systems, iron acquisition from host iron-binding proteins and uptake of ferrous iron, and then outline the potential of targeting iron acquisition in treating CF infections.

SIDEROPHORE-MEDIATED IRON ACQUISITION

Siderophore-mediated iron acquisition has been identified as an important virulence factor for many CF pathogens (Thomas, 2007; Cornelis & Dingemans, 2013). Siderophores

are small iron-chelating molecules secreted by Gram-negative, Gram-positive and fungal micro-organisms. They are generally classed as catecholate-type siderophores that bind ferric iron via hydroxyl groups, hydroxamate-type siderophores that chelate ferric iron via a carbonyl group with an adjacent nitrogen, and mixed-type siderophores that have both catechol- and hydroxamate-binding moieties. *P. aeruginosa*, the predominant pathogen in CF, secretes two siderophores, pyoverdine and pyochelin, in response to iron deprivation (Liu & Shokrani, 1978; Cox & Adams, 1985; Heinrichs *et al.*, 1991). Both pyoverdine and pyochelin biosynthesis and uptake are regulated by the ferric uptake regulator (Fur) protein – the primary controller of iron regulated genes in many Gram-negative bacteria (Ochsner *et al.*, 2002; Michel *et al.*, 2005). The predominant siderophore pyoverdine is a mixed-type siderophore and has a higher affinity for iron than pyochelin (Cox & Graham, 1979). Three structurally different pyoverdines have been identified (Briskot *et al.*, 1986) and are the subject of a recent review (Cézard *et al.*, 2015). Pyoverdine plays a role in *Pseudomonas* pathogenesis in animal models of infection (Meyer *et al.*, 1996; Takase *et al.*, 2000; Xiong *et al.*, 2000). Of particular relevance to CF lung disease is the established role of this siderophore in *P. aeruginosa* biofilm formation (Banin *et al.*, 2005). Pyochelin, a phenolate, is unusual amongst siderophores in having neither catecholate nor hydroxamate groups, and binds Fe^{3+} via the carboxylate group and the phenolic OH group. Pyochelin is produced when iron is less restricted and although it has a lower affinity for iron than pyoverdine, its biosynthesis is a more energy-efficient process (Ravel & Cornelis, 2003; Moon *et al.*, 2008; Cornelis, 2010; Dumas *et al.*, 2013). *P. aeruginosa* has the ability to utilize siderophores from other pseudomonads as well as other bacterial species and fungi. *P. aeruginosa* strains have >30 genes encoding TonB-dependent receptors (TBDRs), which are involved in the uptake of ferrisiderophores (Cornelis & Bodilis, 2009). Receptors for mycobactin and carboxymycobactin (Llamas *et al.*, 2008), ferrichrome and ferrioxamine (Llamas *et al.*, 2006; Cuív *et al.*, 2007; Hannauer *et al.*, 2010), rhizobactin, aerobactin and schizokinen (Cuív *et al.*, 2006), and vibriobactin (Elias *et al.*, 2011) have all been identified. Given the evidence to date, *P. aeruginosa* appears to have the greatest capacity for siderophore piracy in the CF lung microbiome.

Staphylococcus aureus, one of the earlier pathogens to colonize the CF lung, produces two α-hydroxycarboxylate-type siderophores, staphyloferrin A and staphyloferrin B, and expression is regulated by iron through Fur (Beasley *et al.*, 2009). Both siderophores have unique transporters, HtsABC (haem transport system) and SirABC (staphylococcal iron regulated), respectively (Beasley *et al.*, 2009; Cheung *et al.*, 2009). *Staphylococcus aureus* can acquire iron using a variety of xenosiderophores (Table 1), including hydroxamate siderophores, which itself cannot produce, using the conserved ferric hydroxamate uptake system Fhu (Sebulsky *et al.*, 2003, 2004), and catecholate xenosiderophores through the highly conserved staphylococcal siderophore ATP-binding cassette (ABC) transporter SstABCD (Beasley *et al.*, 2011). Iron acquisition strategies of Gram-positive pathogens including *Staphylococcus aureus* are the subject of a recent review by Sheldon & Heinrichs (2015).

Members of the Bcc have the ability to produce up to four siderophores, including ornibactin which is the predominant siderophore produced by *Burkholderia* strains (Darling *et al.*, 1998), and is an important virulence factor for these bacteria to establish and maintain infection (Darling *et al.*, 1998; Sokol *et al.*, 1999, 2000). To date, species known to produce ornibactin under iron starvation include *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Burkholderia vietnamiensis* and *Burkholderia ambifaria* (Meyer *et al.*, 1995; Thomas, 2007; Asghar *et al.*, 2011). More recently, ornibactin gene expression has been reported for the first time in *Burkholderia multivorans* (Denman *et al.*, 2014). There are three known species of this linear hydroxamate siderophore: ornibactin-C4, ornibactin-C6 and ornibactin-C8 produced by Bcc strains (Stephan *et al.*, 1993), and ferrated ornibactin is transported across the outer membrane by a ferric-ornibactin receptor, OrbA. Similar to *Pseudomonas* siderophore biosynthesis, the ornibactin operon is Fur regulated (Agnoli *et al.*, 2006). The second siderophore produced by some strains of the Bcc is pyochelin (Sokol, 1986), and the production of ornibactin and pyochelin correlates with morbidity and mortality in CF (Sokol, 1986; Visser *et al.*, 2004). However, approximately half of Bcc clinical isolates do not produce pyochelin (Darling *et al.*, 1998). Cepaciachelin, a catecholate siderophore first isolated under iron-limiting growth conditions from *Burkholderia ambifaria*, and cepabactin, a cyclic hydroxamate first isolated from *Burkholderia cepacia* ATCC 25416, are also produced by some members of the Bcc (Meyer *et al.*, 1989, 1995; Darling *et al.*, 1998). To date very little is known about the nature of xenosiderophore utilization by Bcc. Our recent study demonstrated that *Burkholderia cenocepacia* J2315 and three clinical isolates of *Burkholderia cenocepacia* cannot utilize pyoverdine from *P. aeruginosa.* Even though these isolates can produce siderophores, growth was severely inhibited in the presence of pyoverdine, presumably due to the high iron-binding capacity of pyoverdine over Bcc siderophores (Tyrrell *et al.*, 2015). Previous studies demonstrated that another member of the Bcc, *Burkholderia cepacia*, is also unable to utilize pyoverdines for iron acquisition (Meyer *et al.*, 1989). Iron acquisition by the Bcc has been comprehensively reviewed by Thomas (2007).

Stenotrophomonas maltophilia, an emerging CF pathogen, produces catechol-type siderophores; however, they have not been fully characterized and their role in infection is unknown (García *et al.*, 2012). Siderophore secretion by some strains of *Achromobacter xylosoxidans* isolated from soil has been reported (Tian *et al.*, 2009); however, no investigations of clinical isolates have been reported. There are no reports of siderophore production by

Table 1. Iron acquisition systems utilized by main CF pathogens

Bacterial species	Endogenous siderophore	Exogenous siderophore	Haem uptake system	Host iron-binding proteins	Ferrous iron uptake system
P. aeruginosa	Pyoverdine Pyochelin	Mycobactin Carboxymycobactin Ferrichrome ferrioxamine Rhizobactin Aerobactin Schizokinen Vibriobactin Pyoverdine	Has Phu	Transferrin Lactoferrin Ferritin	Feo
S. aureus	Staphyloferrin A Staphyloferrin B	Ferrichrome Desferrioxamine-B Aerobactin Coprogen	Isd	Transferrin	*feoABC* locus
Bcc	Ornibactin Pyochelin Cepabactin Cepaciachelin	NR	huvA–hmuSTUV (unconfirmed)	Transferrin Lactoferrin Ferritin	NR
H. influenzae	NR	Ferrichrome (operon)	HpbA		
A. fumigatus	Fusarinine C Triacetylfusarinine C Ferricrocin Hydroxyferricrocin Hexadeydroastechrome	Ferrichrome Coprogen Ferrioxamine B Ferrioxamine E	NR	Transferrin	Ferrireductase FreB Ferroxidase FetC Permease FtrA
C. albicans	Hydroxamate-type Phenolate-type (uncharacterized)	Fusarinine C Triacetylfusarinine C Ferricrocin	Rbt5/Pga7	Transferrin Ferritin	Several ferric reductases

NR, None reported.

H. influenzae; however, comparative genomics has identified an operon for ferrichrome utilization in non-typable *H. influenzae* strains (Morton *et al.*, 2010).

The fungal pathogen *Aspergillus fumigatus* produces four hydroxamate siderophores: fusarinine C and triacetylfusarinine C, which are secreted, and ferricrocin the intracellular iron storage siderophore in hyphae and hydroxyferricrocin the iron siderophore storage in conidial spores (Schrettl *et al.*, 2007; Wallner *et al.*, 2009; Haas, 2012). An additional siderophore-like molecule called hexadeydroastechrome was recently isolated from *Aspergillus fumigatus* and increased virulence when overexpressed in a murine model of infection (Yin *et al.*, 2013). There is significant evidence that iron uptake in *Aspergillus fumigatus* is essential to virulence (Moore, 2013). In particular, siderophore-mediated iron acquisition is vital for *Aspergillus fumigatus* virulence in a murine model of invasive aspergillosis (Schrettl *et al.*, 2004; Hissen *et al.*, 2005) and to the survival of the conidia in macrophages (Schrettl *et al.*, 2010). Siderophore biosynthetic enzymes are also upregulated in conidia internalized by airway epithelial cells (Oosthuizen *et al.*, 2011). *Aspergillus fumigatus* has been reported to utilize xenosiderophores including ferrichrome, coprogen, ferrioxamine B and ferrioxamine E (Petrik *et al.*, 2012). *Candida albicans*, also a frequent colonizer in CF (Chotirmall *et al.*, 2010), has been reported to produce both hydroxamate- and phenolate-type siderophores (Holzberg & Artis, 1983; Ismail *et al.*, 1985); however, siderophore biosynthesis genes have not been identified in the *Candida albicans* genome (Haas, 2003). *Candida albicans* does, however, express a siderophore iron transporter (Sit1) allowing it to transport and utilize ferrichrome-type siderophores including triacetylfusarinine C and ferricrocin from *Aspergillus fumigatus*, and a Sit1 mutant has reduced invasion and penetration in a human epithelium model of infection (Heymann *et al.*, 2002), highlighting the pivotal role of iron acquisition in establishing *Candida albicans* in the CF lung.

HAEM UPTAKE SYSTEMS

Although the majority of haem in the host is bound to intracellular haemoproteins, primarily haemoglobin (Stojiljkovic & Perkins-Balding, 2002), free haem and haemoglobin are released by damaged cells during infection and not surprisingly the successful CF pathogens are all adept at utilizing this iron source. *P. aeruginosa* has the capacity to take up haem from haemoproteins via two systems, Has (haem assimilation system) and Phu (*Pseudomonas* haem uptake; Ochsner *et al.*, 2000). In the Phu system, haemoproteins bind directly to a TBDR and haem is extracted, whereas the Has system uses a secreted haemophore to extract haem from haemoproteins and transport it to a TBDR, HasR. Haem, bound by a periplasmic binding protein, is transported to the cytoplasm by an ABC transporter where it is bound to a haem chaperone, PhuS. It is then degraded by the haem oxygenase HemO and Fe^{2+} is released (Wegele *et al.*, 2004; Kaur *et al.*, 2009). *P. aeruginosa* adapts its iron acquisition strategy depending on available iron, and the Fur protein represses the expression of the Has and Phu systems in response to excess iron (Cornelis & Dingemans, 2013). A recent study demonstrates that in the CF lung, haem uptake is a critical source of iron for *P. aeruginosa* during chronic infection with more efficient utilization of haem by later compared with earlier isolates and consistent expression of the haemoxygenase HemO during prolonged infection (Nguyen *et al.*, 2014). Further evidence of this adaptive response by *P. aeruginosa* is provided in a study by Marvig *et al.* (2014) that demonstrated within-host evolution towards haem utilization, coupled with loss of pyoverdine production in three separate *P. aeruginosa* lineages (Marvig *et al.*, 2014).

Staphylococcus aureus preferentially utilizes haem as a vital source of iron (Skaar *et al.*, 2004b) and the iron-regulated surface determinant (Isd) system, first described in 2002 (Mazmanian *et al.*, 2002, 2003), is now known to involve nine proteins. Four of these proteins (IsdB, IsdH, IsdA and IsdC) form a complex covalently bound to peptidoglycan through which haem is delivered to an ABC transporter comprising IsdE and IsdF (Grigg *et al.*, 2007). Haem is degraded by the haem monooxygenases IsdG and IsdI to release iron (Skaar *et al.*, 2004a). Recently, Hannauer *et al.* (2015) reported the involvement of two additional reductases, NrtA and IruO, in haem iron removal by *Staphylococcus aureus*. The Isd-mediated haem uptake system of *Staphylococcus aureus* was comprehensively reviewed by Hammer & Skaar (2011) and its significance in *Staphylococcus aureus* infections is demonstrated by its requirement for full virulence in several models of pathogenesis, as reviewed by Grigg *et al.* (2010).

The haem acquisition system of *Burkholderia cenocepacia* is reported to be similar to the *huvA–hmuSTUV* system of *Burkholderia pseudomallei* (Shalom *et al.*, 2007), with an operon comprising five genes *bhuRSTUV* predicted to encode the outer membrane haem receptor, a shuttle protein and components of the haem permease (Thomas, 2007). We have reported the uptake of haemin by *Burkholderia cenocepacia* in the absence of siderophore secretion, confirming the ability of these pathogens to exploit this host iron source in the CF lung (Tyrrell *et al.*, 2015).

H. influenzae, unlike the other CF pathogens, has an absolute growth requirement for haem as it cannot synthesis protoporphyrin IX (Panek & O'Brian, 2002). The haem-binding lipoprotein HpbA is required for haem utilization from different sources (Morton *et al.*, 2005, 2009) and is implicated in *H. influenzae* virulence in animal models of infection (Morton *et al.*, 2009).

Much less is known about haem uptake by pathogenic fungi compared with bacteria. The utilization of haem by *Candida albicans* has been described and is mediated by specific receptors such as Rbt5 (Weissman & Kornitzer, 2004; Okamoto-Shibayama *et al.*, 2014). A new haem-binding protein, Pga7, a member of the common fungal extracellular membrane (CFEM) family, has recently been reported in *Candida albicans* and contributes to virulence in a mouse model. *In vitro*, both Rbt5 and Pga7 extract haem from haemoglobin, and haem can be rapidly transferred between these two CFEM proteins (Kuznets *et al.*, 2014). *Candida albicans* also produces the haemolytic molecule mannan which facilitates access to haem-bound iron (Tanaka *et al.*, 1997; Watanabe *et al.*, 1999). Interestingly, *Aspergillus fumigatus* lacks the ability to use haem as an iron source (Eisendle *et al.*, 2003) and its CFEM domain proteins do not play a role in either haem uptake or virulence (Vaknin *et al.*, 2014).

IRON ACQUISITION FROM HOST IRON-BINDING PROTEINS

In the host environment iron is bound to proteins such as ferritin, transferrin and lactoferrin to reduce free iron to $\sim 10^{-18}$ M (Bullen, 1981), and in the CF lung some of these proteins are elevated during infection (Parrow *et al.*, 2013). CF pathogens have developed mechanisms that enable them to acquire iron from host iron-binding proteins during infection. *P. aeruginosa* pyoverdine and pyochelin can displace iron from transferrin (Takase *et al.*, 2000). *P. aeruginosa* also has the ability to release iron from transferrin using elastase (LasB; Wolz *et al.*, 1994), the alkaline protease AprA (Kim *et al.*, 2006) and the endoprotease PrpL (Wilderman *et al.*, 2001). PrpL can also hydrolyse lactoferrin, in addition to other extracellular host proteins, leading to tissue damage and further contributing to the infection process (Wilderman *et al.*, 2001). In the CF lung transferrin and lactoferrin also undergo proteolytic cleavage by human-derived elastases, making iron more readily available for *P. aeruginosa* (Britigan *et al.*, 1993). Ferritin, abundant in the CF lung, is also a source of iron for *P. aeruginosa*. Dehner *et al.* (2013) demonstrated that *P. aeruginosa* can remove iron

from ferritin and transport it into the cell independently of siderophore production (Dehner *et al.*, 2013).

The two *Staphylococcus aureus* siderophores staphyloferrin A and staphyloferrin B are capable of liberating iron from transferrin, which is then carried by the aforementioned ABC transporters Hts and Sir, respectively, into the cell (Beasley *et al.*, 2011). It is also noteworthy in the context of severely ill patients that stress hormones can also facilitate pathogen removal of iron from transferrin and lactoferrin (Freestone *et al.*, 2008). Catecholamine stress hormones form complexes with the ferric iron in these host proteins, resulting in iron reduction and liberation, facilitating enhanced bacterial growth (Sandrini *et al.*, 2010). In a staphyloferrin-deficient *Staphylococcus aureus* strain, the catecholamine-liberated transferrin iron is transported via the Sst ABC transporter (Beasley *et al.*, 2011). Alternatively, *Staphylococcus aureus* is also reported to release iron from transferrin by increasing the production of lactate, resulting in acidification of the surrounding environment (Friedman *et al.*, 2006).

Burkholderia cenocepacia is also capable of exploiting the ferritin iron source in the CF lung by protease cleavage (Whitby *et al.*, 2006). Our studies have shown that *Burkholderia cenocepacia*, in addition to ferritin, also has the ability to utilize lactoferrin and transferrin when siderophore production is unavailable (Tyrrell *et al.*, 2015).

Despite reports of the inhibitory effects of lactoferrin and transferrin on pathogenic fungi (Almeida *et al.*, 2009; Caza & Kronstad, 2013), *Candida albicans* and *Aspergillus fumigatus* can both utilize transferrin bound iron for growth using different mechanisms (Ramanan & Wang, 2000; Hissen *et al.*, 2004). Siderophores are not involved in iron acquisition by *Candida albicans*; rather direct contact of *Candida albicans* with transferrin is required. Iron is then released from transferrin by the activity of the reductase Fre10 and the permease Ftr1 (Ramanan & Wang, 2000). *Aspergillus fumigatus*, similar to the other CF pathogens, uses secreted siderophores to access iron from transferrin (Hissen *et al.*, 2004; Hissen & Moore, 2005). Ferritin use as a sole iron source for fungal pathogens has been best characterized in *Candida albicans* and involves the adhesin Als3 in ferritin uptake with acidification as the probable mechanism for the release of iron (Almeida *et al.*, 2008).

The range of direct and indirect mechanisms used by CF pathogens to access iron from host iron-binding proteins illustrates their ability to exploit this iron pool and undoubtedly facilitates their survival in the CF lung.

FERROUS IRON UPTAKE

Ferrous iron is generally present in very low quantities in the body; however, it is more likely to be present in anaerobic conditions or in microaerobic environments at lower pH which may be relevant in CF lung mucous. A study of sputum from 25 CF patients showed that ferrous iron is abundant in the CF lung and significantly correlates with disease severity (Hunter *et al.*, 2013). In addition, bacterial and fungal pathogens can convert ferric iron to the more accessible ferrous form. *P. aeruginosa* produces redox-cycling phenazines to achieve this, and the soluble Fe^{2+} is transported inside cells via the Feo transport system composed of a permease FeoB and proteins FeoA and FeoC (Cartron *et al.*, 2006). Deletion of the *feoB* gene results in the inability of *P. aeruginosa* to form biofilms and attenuates its virulence (Wang *et al.*, 2011).

Homologues of *feoAB* have been identified in *Staphylococcus* spp. and small putative FeoC-like proteins are encoded within the *feo* gene cluster in these organisms (Cartron *et al.*, 2006). The *Staphylococcus aureus feoAB(C)* locus is upregulated during iron starvation in a Fur-dependent manner (Lin *et al.*, 2012; Ledala *et al.*, 2014).

Recently, Mathew *et al.* (2014) identified a putative iron uptake locus, $ftr_{Bcc}ABCD$, in the genome of *Burkholderia cenocepacia* H111 that is homologous to a recently described ferrous uptake system in *Bordetella pertussis* and *Bordetella abortus* (Brickman & Armstrong, 2012), but it in fact encodes a ferric iron transporter and is not critical for pathogenicity (Mathew *et al.*, 2014).

Candida albicans has at least 17 ferric reductase genes, and the ferrireductase CFL1 plays a significant role in filamentous growth and virulence of *Candida albicans* in a mouse model of infection (Yu *et al.*, 2014). The reduced iron is taken into the cell by a complex of an iron permease with a multicopper oxidase (Knight *et al.*, 2002; Ramanan & Wang, 2000). *Aspergillus fumigatus* also uses reductive iron assimilation involving the ferric reductase FreB to reduce ferric iron, and ferrous iron is then imported using a protein complex consisting of the ferroxidase FetC and the iron permease FtrA (Blatzer *et al.*, 2011).

PATHOGEN INTERACTIONS AND COMPETITION FOR IRON IN CF

Whilst substantial data exist in relation to the iron acquisition mechanisms of individual CF pathogens, the behaviour of these pathogens in the CF lung and how this behaviour modulates during chronic infection is critical to the tackling these infections. In particular, clarifying the dynamics involved in complex intra- and interspecies relationships is a challenge. Studies involving transcriptomic and proteomic data from clinical clonal isolates, real-time quantitative PCR of CF sputa and sequence analysis of target genes in co-culture systems have yielded insights into how these pathogens, particularly *P. aeruginosa*, behave in the clinical context. In two separate studies of chronic isolates from CF patients, *P. aeruginosa* lost the ability to make pyoverdine, but retained the ability to take up ferripyoverdine (De Vos *et al.*, 2001; Smith *et al.*, 2006). A review by Lamont *et al.* (2009) describes the multifaceted and adaptive nature of iron acquisition

by *P. aeruginosa* in the CF lung. More recently, Martin *et al.* (2011) showed that although pyoverdine was detectable in most *P. aeruginosa* infected CF sputa, some sputa were pyoverdine-negative (Martin *et al.*, 2011). Consistent with these findings, Konings *et al.* (2013) subsequently demonstrated using real-time quantitative PCR that genes associated with siderophore-mediated iron acquisition including pyoverdine and pyochelin are expressed at low levels in CF sputum, and both haem and ferrous iron uptake genes were also detected, indicating multiple iron uptake pathways are active but siderophore secretion is downregulated (Konings *et al.*, 2013). This phenomenon was further explored by Andersen *et al.* (2015) in a sequence analysis study which confirmed that in the CF lung environment, many *P. aeruginosa* strains 'cheat' by no longer producing pyoverdine and instead use pyoverdine produced by co-infecting strains (Andersen *et al.*, 2015). Furthermore, the functional pyoverdine receptor is lost when pyoverdine is no longer available – evidence that the *P. aeruginosa* pyoverdine system evolves in response to social interactions (Andersen *et al.*, 2015).

Interactions between *P. aeruginosa* and other pathogenic species also have implications for iron acquisition in the CF lung. An early study by McKenney *et al.* (1995) showed that the presence of *P. aeruginosa* supernatant enhances siderophore production by *Burkholderia cepacia*, indicating that the pathogenesis of *Burkholderia cepacia* can be modulated by *P. aeruginosa*. Consistent with those data, we have recently demonstrated that pyoverdine from *P. aeruginosa* increases the expression of ornibactin synthesis genes by *Burkholderia cenocepacia* with a concomitant inhibition of growth (Tyrrell *et al.*, 2015). Conversely, Weaver & Kolter (2004) demonstrated using microarray analysis that *Burkholderia*-conditioned medium and ornibactin induced genes involved in iron regulation in *P. aeruginosa* strains. However, *P. aeruginosa* is unable to utilize ornibactin for iron acquisition (Weaver & Kolter, 2004). Bakkal *et al.* (2010) have reported that bacteriocins produced by *Pseudomonas* and *Burkholderia* strains have intra- and interspecies bacteriocin-like inhibition ability within the CF lung (Bakkal *et al.*, 2010). Some of these bacteriocins, such as the *Pseudomonas* pyocin, share the same receptors as siderophores (Denayer *et al.*, 2007; Elfarash *et al.*, 2014) and therefore interfere with iron acquisition of their target host. It has also been demonstrated that *P. aeruginosa* can lyse *Staphylococcus aureus* using PqsA, a coenzyme ligase, to gain access to internalized iron (Mashburn *et al.*, 2005). *Staphylococcus aureus*, however, can also compete with *P. aeruginosa* for free iron (Mashburn *et al.*, 2005; Harrison *et al.*, 2008). Nguyen & Oglesby-Sherrouse (2015) recently reported that iron depletion increases the ability of *P. aeruginosa* to suppress the growth of *Staphylococcus aureus*, and subsequently Filkins *et al.* (2015) demonstrated that *P. aeruginosa* requires both of its major siderophores to kill *Staphylococcus aureus* in a CF bronchial epithelial co-culture model (Filkins *et al.*, 2015) – all evidence that iron plays a central role in modulating interactions between *P. aeruginosa* and *Staphylococcus aureus* in the CF lung. Evidence also suggests that complex bacterial–fungal interactions occur in the CF lung. An early report demonstrated that *P. aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit the growth of *Aspergillus fumigatus* (Kerr *et al.*, 1999). A recent study confirmed that the inhibition by phenazines was due to the production of reactive oxygen species, but that the chelating properties of 1-hydroxyphenazine induced iron starvation (Briard *et al.*, 2015). An interesting study in the context of CF lung infection has shown that CF *P. aeruginosa* isolates are more inhibitory to *Aspergillus fumigatus* growth and biofilm formation than non-CF isolates, and that non-mucoid isolates are the most inhibitory (Ferreira *et al.*, 2015). Culture filtrates from these isolates follow the same pattern of inhibition, and notably culture filtrates from biofilm-grown *P. aeruginosa* were more inhibitory to *Aspergillus fumigatus* biofilms than filtrates from planktonic cultures (Ferreira *et al.*, 2015) – clear evidence that adaptive changes in *P. aeruginosa* growth impact on the interactions with co-infecting organisms. There is also evidence that *Aspergillus fumigatus* responds to *P. aeruginosa* exoproducts with the demonstration that *Aspergillus fumigatus* metabolizes phenazines secreted by *P. aeruginosa* to 1-hydroxyphenazines and that 1-hydroxyphenazine stimulates the secretion of the siderophore triacetylfusarinine C by *Aspergillus fumigatus* (Moree *et al.*, 2012). *P. aeruginosa* phenazines also alter *Candida albicans* colony morphology and cellular respiration, and inhibit the growth and biofilm formation by this fungus, but whether these effects are due to the chelation properties of phenazines is unknown (Morales *et al.*, 2013). Further insight into the interactions between these two pathogens is provided by a study from Chen *et al.* (2014) which demonstrates that *P. aeruginosa* phenazines induce alcohol production by *Candida albicans* which stimulates *P. aeruginosa* biofilm formation, but also alters the profile of phenazines produced by *P. aeruginosa* in favour of those that are most inhibitory towards *Candida albicans*. Whether this effect is due to the chelating properties of phenazines leading to *Candida albicans* iron starvation has not been established; however, given the effect of phenazines on the iron homeostasis of *Aspergillus fumigatus* (Briard *et al.*, 2015), it is likely that it is at least a contributing factor. Taken together, these reports highlight the complexity of interspecies competition for iron and survival in the CF lung, and possible mechanisms by which *P. aeruginosa* dominates in this environment.

NOVEL THERAPEUTIC STRATEGIES TARGETING IRON ACQUISITION IN CF PATHOGENS

The absolute requirement for iron by CF pathogens has led to investigations of iron chelation therapy in an effort to limit its availability and compromise pathogen growth and survival in the host. Moreau-Marquis *et al.* (2008)

demonstrated reduced *P. aeruginosa* biofilm growth on epithelial cells treated with an iron chelator and subsequently demonstrated (Moreau-Marquis *et al.*, 2009) tobramycin in combination with iron chelators can eliminate *P. aeruginosa* biofilms. In a recent study, the same group have shown that a combination of hypocyanite and lactoferrin enhances the ability of tobramycin and aztreonam to eliminate *P. aeruginosa* biofilms on lung epithelial cells (Moreau-Marquis *et al.*, 2015). Data from Reid *et al.* (2009) indicate that iron chelation enhances the efficacy of tobramycin therapy in a low oxygen environment, such as that which pertains in areas of the CF lung. Taken together, these data provide strong evidence for iron chelation to be an effective adjunctive therapy in treating *P. aeruginosa* lung infections. Treatment with the host iron-binding protein lactoferrin reduces both the epithelial invasion of *Burkholderia cenocepacia* and the inflammatory response of CF bronchial epithelial cells to *Burkholderia cenocepacia* biofilm (Berlutti *et al.*, 2008). Whether these effects are the result of iron chelation or an alternative mechanism is unknown. Lactoferrin has been shown to interact with the cable pilus of *Burkholderia cenocepacia* (Ammendolia *et al.*, 2010), which may interfere with cell attachment. The iron chelator desferasirox in combination with liposomal amphotericin B in a murine model of invasive aspergillosis resulted in a significant decrease in fungal burden and enhanced survival compared with placebo-treated mice (Ibrahim *et al.*, 2010). However, other dual- and triple-therapy combinations with desferasirox worsened outcomes (Ibrahim *et al.*, 2011). Therefore, it as yet unclear whether combining iron chelators with conventional antifungals will have any future in the clinical treatment of invasive aspergillosis.

Despite the multifaceted capacity for iron acquisition demonstrated by all of the main CF pathogens (summarized in Table 1) and the redundancy of their iron-scavenging systems, targeting siderophore-mediated iron uptake systems has proven to have significant potential as a bacteriostatic or antimicrobial strategy. One approach is the inhibition of the catalytic mechanisms of siderophore biosynthesis. Non-ribosomal protein synthetase (NRPS) enzymes have no human homologues and the catalytic mechanisms involved in the biosynthesis of many siderophores have been characterized, which facilitates the targeting of siderophore biosynthesis as a therapeutic strategy. Imperi *et al.* (2013) screened a library of bioactive compounds for pyoverdine-inhibitory compounds. A compound, flucytosine, which inhibits PvdS, a promoter of virulence genes including pyoverdine synthesis genes, was identified and flucytosine reduced *P. aeruginosa* pathogenicity in a mouse model (Imperi *et al.*, 2013). Baulamycin A, a broad-spectrum antibiotic produced by a *Streptomyces* spp., acts as a competitive inhibitor of the *Staphylococcus aureus* SbnE synthetase, involved in the NRPS-independent synthesis of staphyloferrin B, and impedes the growth of *Staphylococcus aureus* under iron restriction (Tripathi *et al.*, 2014). This report demonstrates the enormous potential of targeting siderophore biosynthesis pathways using these natural baulamycins, which can be further modified to enhance efficacy and specificity. Inhibitors of siderophore biosynthesis targeting both NRPS enzymes and those involved in NRPS-independent pathways of siderophore biosynthesis have been extensively reviewed by Lamb (2015). An alternative approach has been the genetic engineering of the NRPSs to synthesize novel siderophores–a strategy which has been applied to the production of novel pyoverdines (Calcott & Ackerley, 2014; Calcott *et al.*, 2014). In addition to targeting siderophore iron acquisition, the inhibition of the final step in liberating iron from haem has also been investigated. Using a computer-aided drug design approach, inhibitors that bind to the haem pocket of the HemO enzyme have been identified, and shown to have *in vitro* activity against *P. aeruginosa* clinical isolates and *in vivo* activity in the *Caenorhabditis elegans* host–pathogen model of infection (Hom *et al.*, 2013).

There are no licensed vaccines currently available for any of the CF pathogens discussed above. Attempts have been made to develop vaccines based on iron acquisition receptors, although success to date has been limited and only those targeting *Staphylococcus aureus* have reached clinical trials. A vaccine against the *Staphylococcus aureus* haemoglobin receptor IsdB, V710, generated promising data from phase I and IIa trials; however, a lack of efficacy and safety concerns emerged in a subsequent trial (Fowler *et al.*, 2013). In a recent study, vaccination with a three-component vaccine including antigenic regions of IsdB reduced bacterial load and increased survival in a murine model of *Staphylococcus aureus* infection (Delfani *et al.*, 2015). Another vaccine based on the ferric hydroxamate receptor FhuD2 only offered partial protection against staphylococcal infection in murine models (Mishra *et al.*, 2012; Mariotti *et al.*, 2013).

One of the most successful therapeutic approaches to date involving bacterial iron acquisition systems has been the exploitation of these pathways to deliver antibiotic–siderophore conjugates to target cells. This concept is based on the naturally occurring sideromycin antibiotics that mimic siderophores to gain intracellular access (Braun *et al.*, 2009). Over the past three decades, numerous conjugates linking a siderophore or a siderophore mimic to different antibiotics via a linker molecule have been synthesized with varying degrees of antimicrobial efficacy (Möllmann *et al.*, 2009; Wencewicz *et al.*, 2009; Zeng *et al.*, 2012; Fardeau *et al.*, 2014). This approach, known as the 'Trojan horse' strategy, has been the subject of several reviews (including those by de Carvalho & Fernandes, 2014; Górska *et al.*, 2014; Mislin & Schalk, 2014). Challenges to the design of these conjugates include optimal linker design to avoid interference with siderophore receptor binding and the development of resistance due to the loss of components of the siderophore uptake system (Minnick *et al.*, 1992). Competition with native siderophores was also reported in a recent mouse model of *P. aeruginosa* infection treated with a siderophore-conjugated monobactam

(Tomaras *et al.*, 2013). A recent siderophore sulfactam conjugate BAL20072 developed by Basilea Pharmaceutic has shown significant promise against multidrug-resistant Gram-negative isolates including *P. aeruginosa* and Bcc spp. (Page *et al.*, 2010; Hofer *et al.*, 2013; Landman *et al.*, 2014), and is currently in phase 1 clinical trials. Another promising conjugate in development is S-649266 from Shionigi, which is a catechol-substituted siderophore conjugated to cephalosporin with significant activity against multidrug-resistant Gram-negatives including *P. aeruginosa*. Phase II trials of this drug are currently under way (ClinicalTrials.gov ID: NCT02321800). In addition to antimicrobial siderophore conjugates, haem uptake systems also have the potential to be targeted with antibiotic–porphyrin conjugates (Stojiljkovic *et al.*, 2001). Natural and synthetic porphyrins exhibit a range of light-dependent and light-independent antibacterial activities, exhibit low toxicity *in vivo*, and are amenable to chemical modification, making them ideal compounds for targeting microbial pathogens (Stojiljkovic *et al.*, 1999). Given the increasing literature on the structures and pathways involved in bacterial iron acquisition, it is becoming more likely that a successful synthetic analogue drug delivered to the cell via an iron uptake mechanism will eventually come to market.

CONCLUSION

Iron acquisition is a multifaceted and dynamic process for CF pathogens which is essential for colonization and infection in the competitive environment of the CF lung. Siderophore-mediated iron uptake is an important component of this process, with the most prominent CF pathogen, *P. aeruginosa*, capable of extensive siderophore piracy. The deployment of alternative iron acquisition systems, including the uptake of ferrous iron and the scavenging of iron from host iron acquisition proteins, further enhances the ability of pathogens to establish infection and poses additional challenges to targeting iron acquisition as a therapeutic strategy. Nonetheless, the urgent need to combat antibiotic-resistant lung infections has led to a renewed focus on novel approaches to this problem and iron acquisition pathways remain in the spotlight.

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