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Minireview

The evolving dynamics of the microbial community in the cystic fibrosis lung

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Summary

The cystic fibrosis (CF) lung is a niche colonized by a diverse group of organisms, with a more limited number of species including *Pseudomonas aeruginosa* dominating in adult patients. Whether all members of this microbial community play a direct or indirect role in pulmonary decline has yet to be fully elucidated, but investigations of their interactions with both co-colonizing species and with host cells are beginning to shed light on their virulence potential. It is also emerging that some microbial species within this community adapt as chronic infection is established to survive the hostile environment of the lung, to minimize host clearance and to resist therapeutic intervention. This review highlights the recent developments in CF microbiology focusing on the cooperative, competitive and adaptive interactions of established and emerging pathogens in the lung microbiome.

Introduction

Cystic fibrosis (CF) is a recessive genetic disease caused by the loss or dysfunction of a protein responsible for transmembrane chloride and water transport, known as the CF transmembrane conductance regulator (CFTR). This defect leads to dehydrated mucous secretions and low airway surface pH, which facilitates colonization and infection with a range of bacterial species and a concomitant deterioration of lung function, ultimately leading to the premature death of these patients. Since CF was first described (Andersen, 1938), it has taken almost 75 years for the first corrective therapy targeting the underlying defect in the CFTR to appear on the market. Despite the significant breakthrough of the approval of the CFTR-modulating drug ivacaftor, it is currently only licensed for use in approximately 5% of CF patients carrying the Gly551Asp mutation (Ong and Ramsey, 2013; O'Reilly and Elphick, 2013). As the CF community awaits development of additional CFTR corrective therapies, they continue to rely on the effective management of their recurrent infections to retain lung function and improve life expectancy.

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CF lung microbiome

The application of technologies such as high-throughput sequencing and microarrays to interrogate 16S rRNA gene sequences has proven useful in defining the complex nature of the microbial community in the CF lung (Claesson *et al.*, 2010; Klepac-Ceraj *et al.*, 2010; Guss *et al.*, 2011; Blainey *et al.*, 2012; Zhao *et al.*, 2012). Despite the diversity, the bacterial species with established clinical relevance in CF lung disease are relatively few and the potential contribution of a large number of species remains undefined. Interactions of clearly defined CF pathogens with the host have been recently reviewed (Callaghan and McClean, 2012). What is emerging from longitudinal studies however, is that the CF microbiome evolves with age (Zhao *et al.*, 2012), and the diversity of the airway bacterial community is diminished in older patients, where members of the Pseudomonadaceae family dominate (Cox *et al.*, 2010). The role of chronic *Pseudomonas aeruginosa* infection in lung function decline has been well established. More recently however, it has emerged that the *P. aeruginosa* strain population in CF is highly diverse. In a collection of 167 clonal isolates from a single patient, a wide diversity of phenotypes was represented even within isolates of the same colony morphotype from the same sample. This within-patient diversity may be a factor in the dominance of this species in CF (Workentine *et al.*, 2013).

Another significant group of pathogens is the *Burkholderia cepacia* complex (Bcc), currently comprising 18 genotypically diverse but phenotypically similar Gram-negative species (Vanlaere *et al.*, 2008; 2009; Peeters

et al., 2013). Bcc species are notoriously antibiotic resistant and are associated with a fatal pneumonia known as 'cepacia syndrome' in some patients. The pathogenic mechanisms of this group of pathogens have been previously reviewed (McClean and Callaghan, 2009; Sousa *et al*., 2011). *Burkholderia cenocepacia* and *B. multivorans* are the most clinically prevalent species of the complex with other species from this group including *B. dolosa* emerging as potential contributors to CF morbidity and mortality (Kalish *et al*., 2006). Patient to patient transmission of Bcc organisms has lead to outbreaks in CF centres; however, there is increasing evidence that acquisition of environmental isolates also causes clinical infection. Many species of the Bcc have potential biotechnological applications including biocontrol bioremediation, and plant growth promotion however their use is banned given their potential to colonize CF and immunocompromised individuals (Vial *et al*., 2011). In 2002, an epidemic Bcc strain was isolated from soil (LiPuma *et al*., 2002), and in a later study of Bcc species from different environments, Baldwin and colleagues (2007) demonstrated that > 20% of 381 clinical isolates were indistinguishable from those in the environment. Given the adaptability of these species, it is highly likely that their commercial potential will remain restricted unless non-pathogenic species can be identified and fully characterized. Another Gram-negative pathogen gaining increasing attention is *Stenotrophomonas maltophilia* as chronic infection with this species is an independent predictor of pulmonary exacerbation. However, there is as yet no evidence of a clinical benefit derived from treating these infections (Amin and Waters, 2012). Furthermore, in a Danish study, the rate of decline in *S. maltophilia*-infected patients was the same as observed 3 years before the patients became chronically infected, suggesting that this species may not contribute significantly to CF lung disease (Dalboge *et al*., 2011). *Achromobacter xylosoxidans* is another species capable of establishing chronic infection in CF but in an Italian cohort of patients, it was not associated with any significant clinical impact (Lambiase *et al*., 2010). *Haemophilus influenzae* is associated with early stage CF but its abundance diminishes with age (Cox *et al*., 2010). Another emerging pathogen in CF is the genus *Pandoraea* of which there are nine named species. A *Pandoraea apista* outbreak and concomitant decline in lung function was reported in Danish patients (Jorgensen *et al*., 2003). Investigations on the virulence potential of *Pandoraea* isolates have demonstrated that they are capable of inducing a potent immune response *in-vitro* (Caraher *et al*., 2008) and *P. pnomenusa* invades lung epithelial cells and translocates through the epithelium (Costello *et al*., 2011). Structural analysis of the lipopolysaccharide (LPS) from *P. pulmonicola* has revealed unique features that may contribute to the virulence of some strains (Di Lorenzo *et al*., 2012).

Gram-positive species also feature in early CF lung disease with *S. aureus* frequently isolated from paediatric CF patients and many of these isolates are methicillin resistant. In particular, the small colony variants (SCVs) of this species are independently associated with poorer clinical outcomes in CF paediatric patients (Pillarisetti *et al*., 2011; Wolter *et al*., 2013). The three Streptococcal species *S. constellatus*, *S. intermedius* and *S. anginosus* known as the *Streptococcus milleri* group (SMG) have been associated with exacerbations in CF and, at the onset of the exacerbation, are the numerically dominant pathogens in over a third of these patients (Sibley *et al*., 2008a). Significantly, these patients respond to specific antimicrobial therapy indicating that SMG species warrant greater attention. There has been increasing recognition of the presence of a diverse range of anaerobic species predominantly from the *Prevotella*, *Veillonella*, *Propionibacterium* and *Actinomyces* generas in the CF lung (Tunney *et al*., 2008; Fodor *et al*., 2012) but with no clear evidence of an impact on lung disease for any of these species. Of note, *Prevotella intermedia* is capable of inducing a host response in a murine model and is more cytotoxic *in vitro* compared with anaerobic *P. aeruginosa* but not aerobic *P. aeruginosa* (Ulrich *et al*., 2010). A study that applied parallel pyrosequencing to identify changes in the CF airway microbial community revealed that anaerobic species numbers were not significantly reduced in response to antibiotic therapy (Fodor *et al*., 2012). Clinical trials involving therapies specifically targeted at this population are required in order to define the significance of anaerobes in CF. Although bacterial pathogens garner most of the attention of CF clinicians and microbiologists (summarized in Table 1), fungal species, predominantly *Aspergillus fumigatus* (Delhaes *et al*., 2012; Speirs *et al*., 2012) also feature in the CF microbiome as do respiratory viruses which are frequently associated with severe pulmonary exacerbations in young patients (Asner *et al*., 2012).

Despite changes in microbial burden, the overall community composition remains relatively stable in adult CF patients through antibiotic treatment and clinical exacerbations (Fodor *et al*., 2012). This relative stability suggests that the dominant pathogens adapt to the host environment and compete and cooperate effectively with co-colonizers to establish a community that is resilient to antimicrobials and host responses. Functional biological studies are beginning to uncover some of these interactions and determine the potential contributions of these organisms to CF lung disease.

Adaptive responses of CF pathogens

Successful pathogens in CF overcome the challenges of hypoxic and anoxic niches in airways, nutrient limitation,

Table 1. Bacterial pathogens in the CF lung.

Established CF bacterial pathogens	Adaptation in the CF lung	Primary features of established CF bacterial pathogens	Reference
P. aeruginosa	Low Oxygen	Switch to mucoid phenotype, increased biofilm formation, reduced virulence.	Bragonzi *et al.*, 2009; Lore *et al.*, 2012
B. cenocepacia	Nutrient deficiency	Switch to non-mucoid phenotype, increased invasion of epithelial cells, growth under O_2 limitation.	Madeira *et al.*, 2011;Zlosnik *et al.*, 2011; Sass *et al.*, 2013
B. multivorans	Competition and co-operation	Switch to non-mucoid phenotype, reduced motility, increased biofilm formation.	Silva *et al.*, 2011; Zlosnik *et al.*, 2011
S. aureus	Antimicrobial therapy	Switch to small colony variants (SCVs), reduced capsule formation, increased biofilm formation.	Hirschhausen *et al.*, 2013
H. influenzae	Host response	Associated primarily with early stage CF.	Cox *et al.*, 2010
Emerging CF bacterial pathogens			
Pandoraea spp. (*P. apista*, *P. pnomenusa*)		Some species induce potent pro-inflammatory responses *in-vitro* and epithelium (*P. pnomenusa*)	Caraher *et al.*, 2008; Costello *et al.*, 2011
Anaerobes (*P. intermedia*)		Many anaerobes highly antibiotic resistant – may facilitate persistence	Fodor *et al.*, 2012
Streptococcus milleri group (SMG) and *Stenotrophomonas maltophilia*		Associated with pulmonary exacerbations but role in CF lung disease unclear	Sibley *et al*, 2008a; Amin and Waters, 2012
Achromobacter xylosoxidans		Establishes chronic infection but impact undefined	Lambiase *et al.*, 2010

frequent antibiotic exposure and the multifaceted innate host response (Fig. 1). Transcriptomic, proteomic and metabolomic studies have provided a valuable insight into the range of adaptive responses of CF pathogens when they transition to the lung. *Pseudomonas aeruginosa* strains with high mutation rates are frequently isolated from the CF lung (Oliver *et al.*, 2000) indicative of an organism striving to adapt and thrive in a challenging environment. More recently, Traverse and colleagues (2013) demonstrated that the multiple adaptive alleles responsible for *Pseudomonas* and *Burkholderia* adaptation were found in specific loci with considerable overlap between the species, including genes involved in biofilm formation and iron acquisition. The main features of chronic *P. aeruginosa* infection isolates include reduced virulence (Lore *et al.*, 2012), a switch to a mucoid phenotype triggered by a mutation in the transcriptional regulator *mucA* (Bragonzi *et al.*, 2009), increased non-alginate exopolysaccharide production and enhanced capacity for biofilm formation (Huse *et al.*, 2013). Throughout chronic infection, mucoid isolates of *P. aeruginosa* maintain biofilm-forming capacity in addition to a relatively stable transcriptional profile compared with non-mucoid isolates (Lee *et al.*, 2011). Biofilm production also facilitates the development of morphotypic variants of *P. aeruginosa* (Woo *et al.*, 2012), further perpetuating the selective and adaptive process. In contrast to *P. aeruginosa*, there is evidence that *B. cenocepacia* switches from mucoid to non-mucoid in chronically infected patients (Zlosnik *et al.*, 2008; 2011; Zlosnik and Speert, 2010; Silva *et al.*, 2011) and isolates with a non-mucoid phenotype are associated with a decline in lung function (Zlosnik and Speert, 2010; Silva *et al.*, 2011; Zlosnik *et al.*, 2011), indicative of differing adaptive strategies for these two important CF pathogens. Transcriptomic and phenotypic analysis of *B. multivorans* sequential isolates from a CF patient, where the organism had switched from mucoid to non-mucoid phenotype, revealed loss of exopolysaccharide (EPS) production, reduced motility and increased biofilm formation (Zlosnik and Speert, 2010; Silva *et al.*, 2011; Zlosnik *et al.*, 2011). In another study of *B. cenocepacia* sequential clonal isolates taken from a patient over a 3.5 year period prior to death from cepacia syndrome, we have demonstrated that later isolates are more invasive of epithelial cells and disrupt tight junction integrity to a greater extent compared with early isolates (Madeira *et al.*, 2013), both responses having the potential to alter the course of infection. A recent study has also determined that *S. aureus* persistence in the CF lung is associated with a complex adaptive process that includes a switch to SCVs, reduced capsule formation, in addition to increased biofilm formation and antibiotic resistance (Hirschhausen *et al.*, 2013).

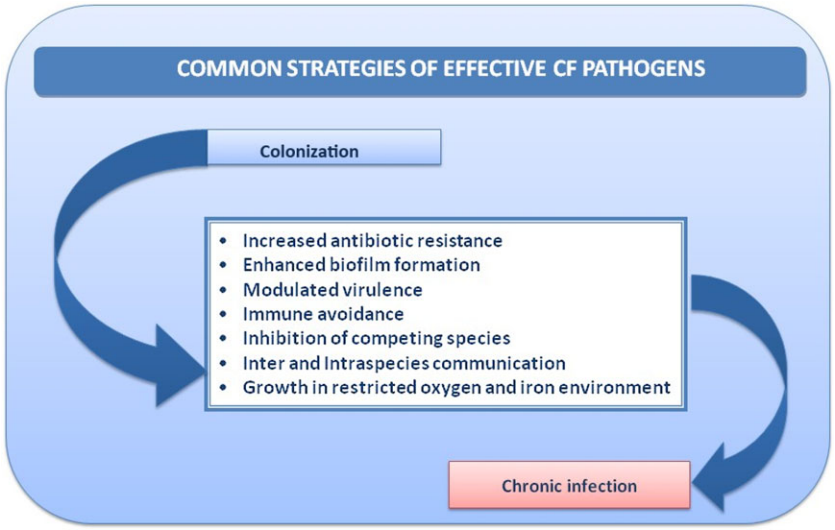

Fig. 1. Adaptive strategies of successful CF pathogens.

Adaptation to nutrient and oxygen deprivation

In addition to the phenotypic changes, biofilm development and altered interactions with host cells, which confer significant advantages to lung pathogens, metabolic adaptations are also evident which facilitate survival in a nutrient depleted niche. Growth complementation between auxotrophic and prototrophic isogenic strains of *P. aeruginosa* has been observed in isolates from chronically infected patients (Qin *et al*., 2012), indicative of the benefits of metabolic diversity between strains. A key nutrient for pathogen survival is iron, and *P. aeruginosa* utilizes several strategies to acquire iron. (Konings *et al*., 2013), using RT-qPCR of sputum RNA, have shown that a range of iron acquisition mechanisms are active in chronic CF infection including siderophore mediated uptake, heme uptake and ferrous iron uptake. Proteomic and genomic studies of clonal *B. cenocepacia* isolates have also confirmed an increased capacity for iron acquisition by this species during chronic infection (Madeira *et al*., 2011; Mira *et al*., 2011) but with a reduced reliance on siderophore-mediated iron acquisition (Madeira *et al*., 2013).

CF pathogens adapt to grow and form biofilms under the hypoxic and anoxic conditions of lung niches with increased polysaccharide secretion by *P. aeruginosa* under hypoxia and upregulation of the outer membrane protein OprF, required for growth and biofilm formation in oxygen-limiting conditions (Hoboth *et al*., 2009). Two recent studies have demonstrated that aerobic *B. cenocepacia* can also survive in very low concentrations of oxygen (Pessi *et al*., 2013; Sass *et al*., 2013). Comparative expression analysis of the aerobic *B. cenocepacia* species identified the expression of genes which allows this organism to grow under low oxygen conditions, a significant advantage for the colonization of lower niches in the lung (Sass *et al*., 2013). Furthermore, in an oxygen-limited environment, *B. cenocepacia,* similar to *P. aeruginosa*, produces larger amounts of biofilm and is more antibiotic resistant (Pessi *et al*., 2013).

Adaptation facilitated by quorum sensing

Quorum sensing (QS) plays an important role in bacterial communication in the CF lung as in other niches, facilitating coordinated activity that enhances survival in a hostile environment. It has been known for over a decade that biologically active levels of QS molecules are secreted by bacteria in the CF lung (Middleton *et al*., 2002; Chambers *et al*., 2005), and more recently, the detection and quantification of *P. aeruginosa* QS molecules in 45 out of 47 CF sputa indicates a potential role for QS molecules in assessing infection status (Struss *et al*., 2013). *Pseudomonas aeruginosa* has at least three QS systems, all of which regulate biofilm formation and exoenzyme production among other cellular activities. The Las and Rhl systems produce *N*-acyl homoserine lactones (AHL) signalling molecules and the *Pseudomonas* quinolone signal (PQS) system employs 2-alkyl-4-quinolone signalling (Aqs). A type VI secretion system and membrane vesicles facilitate the delivery of effector proteins from *Pseudomonas* to target organisms (Tashiro *et al*., 2013). Intraspecies signalling is best evidenced by the activity of biofilm formation whereby *P. aeruginosa* AHLs secreted by biofilms are detected by adjacent microcolonies which respond to establish new biofilms (Flickinger *et al*., 2011) and AHL mutants or so called 'cheaters' which are frequently isolated from CF patients (D'Argenio *et al*., 2007) may benefit from the signalling molecules of cooperator cells. Increasing numbers of AHL QS mutants are isolated during the course of chronic *P. aeruginosa* infection and

the reduced density biofilms associated with these particular strains are more susceptible to antibiotics (Popat *et al*., 2012). However, these mutants have a growth advantage over wild-type strains (D'Argenio *et al*., 2007) and there is also evidence to suggest that *P. aeruginosa* isolates retain the Rhl system longer than the Las system (Bjarnsholt *et al*., 2010). In addition, a recent study has demonstrated that CF isolates of *P. aeruginosa* produce high levels of the PQS (Guo *et al*., 2013), further evidence that bacterial communication by this species in the CF lung is not confined to a single QS system. The deployment of QS systems allows these pathogens to engage in complex intraspecies and interspecies interactions, some of which are competitive and others of which are cooperative in nature. Members of the Bcc also have at least three AHL-mediated QS systems. The cepIR system is conserved across the complex, the cciIR system is present in the epidemic *B. cenocepacia* strains and the BviIR system is present in *B. vietnamiensis* (Gotschlich *et al*., 2001; Conway and Greenberg, 2002; Baldwin *et al*., 2004). In addition, *B. cenocepacia* has a fatty acid signal-based QS system involving a diffusible signal factor present in CF sputum (Boon *et al*., 2008) which may increase antimicrobial resistance of *P. aeruginosa* biofilms (Twomey *et al*., 2012). QS also plays a key role in Bcc biofilm formation, however, in contrast to *P. aeruginosa* isolates, low levels of QS mutants are detected in chronic Bcc infection (McKeon *et al*., 2011), again highlighting some divergent adaptive strategies among pathogens striving to survive in the same niche.

Adaptation to therapeutic interventions

Adaptive responses to therapies are also evident among CF pathogens and include the selection of antibiotic-resistant mutants or phenotypic alterations which facilitate bacterial survival. In *P. aeruginosa*, the two-component regulatory system ParR-ParS plays a key role adaptive resistance and regulates resistance to at least four classes of antibiotics through the activation of the MexXY-OprM efflux pump, down regulation of porin expression and through activation of the LPS modification operon (arnBCADTEF) (Muller *et al*., 2011). Treatment of *S. aureus* by the antibiotic trimethoprim–sulfamethoxazole is also likely to result in SCV formation which is associated with poorer outcomes in pediatric patients (Wolter *et al*., 2013). To further compound the issue of antimicrobial resistance, SCVs of *P. aeruginosa* that develop following prolonged antibiotic exposure have increased levels of EPS and enhanced biofilm formation (Lory *et al*., 2009). Phenotypic analysis of early versus late clonal isolates of *B. cenocepacia* also demonstrates increased antibiotic resistance in later isolates (Coutinho *et al*., 2011), a finding which has been further substantiated with proteomic (Madeira *et al*., 2011) and genomic studies (Mira *et al*., 2011). Bacteria also adapt to therapies that do not specifically target microbes. Mannitol as an inhaled osmolyte therapy in CF also has the potential to impact on the transcription of several virulence traits of *B. multivorans* including upregulation in flagella, siderophore, oxidative stress protein and phospholipase expression in addition to enhancing biofilm formation and epithelial invasion of this species (Denman *et al*., 2013).

Adaptation to the host response

One of the most significant features of CF bacterial host interactions is the ability of the pathogens to circumvent or modulate the multifaceted host response mounted following colonization.

Strategies for bacterial survival include the alteration of structural features or specific avoidance mechanisms. *Pseudomonas aeruginosa* loses flagellar motility during chronic infection thereby impairing recognition by the immune system and clearance by alveolar macrophages (Amiel *et al*., 2010; Patankar *et al*., 2013). Alterations to another Toll-like receptor (TLR) ligand, the lipid A moiety of LPS have also been reported. *Pseudomonas aeruginosa* isolated from chronically infected CF patients produce lipid A modified to a hexa-acylated form involving the addition of aminoarabinose, palmitate and phosphoethanolamine. This modified LPS has more potent immunostimulatory activity than the penta-acylated form; however, the addition of positively charged aminoarabinose reduces the affinity of *P. aeruginosa* lipid A for polymyxin and for cationic antimicrobial peptides (Moskowitz *et al*., 2004; Fernandez *et al*., 2013). It has also been suggested that the acylation pattern of lipid A from *B. cenocepacia* and *B. multivorans* are responsible for the proinflammatory responses elicited by LPS from these species (De Soyza *et al*., 2004) and the O-antigen of *B. cenocepacia* is also capable of inflammasome activation (Kotrange *et al*., 2011).

Over the past decade, investigations have uncovered multifaceted strategies of immune avoidance by the pathogens that dominate in CF lung disease. Members of the Bcc complex can translocate across polarized epithelial layers having disrupted tight junction complexes (Kim *et al*., 2005; Duff *et al*., 2006). Bcc species can also invade epithelial cells with cellular adhesion involving glycosphingolipids on the epithelial surface (Mullen *et al*., 2010). Epithelial invasion by *P. aeruginosa* is also enhanced by binding of flagella to asialo GM1 (Feldman *et al*., 1998) which is upregulated in CF epithelia (Muir *et al*., 2004). Internalized *B. cenocepacia* and also *P. aeruginosa* can escape intracellular clearance mechanisms. *Pseudomonas aeruginosa* uses the type III secretion system ExoS protein to avoid acidified compartments

within epithelial cells (Heimer *et al*., 2013). A type IV secretion system in the ET12 epidemic clonal lineage of *B. cenocepacia* (ptwT4) has been implicated in the intracellular survival and replication of this pathogen in both epithelial cells and macrophages (Sajjan *et al*., 2008). *Burkholderia cenocepacia* having escaped from the endocytic system trigger the autophagy pathway but block autophagosome completion and migrate to the endoplasmic reticulum where they replicate (Sajjan *et al*., 2008; Al-Khodor *et al*., 2014). Macrophage survival by this species has been further defined, and in addition to the delayed acidification of vacuoles in these cells (Flannagan and Valvano, 2008) and altered recruitment of the NADPH oxidase system at the phagosomal membrane (Keith *et al*., 2009), fusion of endosome to lysosome in *B. cenocepacia*-infected macrophages is prevented by the impaired activation of the Rab7 protein (Huynh *et al*., 2010). *Burkholderia multivorans* is also capable of survival within human macrophages although there is evidence of phagosomal maturation (Schmerk and Valvano, 2013), suggesting that Bcc members adopt differing strategies of survival in the macrophage. Phenotypic switching of *S. aureus* to SCV phenotype also facilitates immune avoidance (Tuchscherr *et al*., 2011) and potentially contributes to the persistence of this organism in some CF patients.

Secretory products of pathogens also contribute to the survival strategy. More recently, the secretion of alkaline protease by *P. aeruginosa* has been linked to the activation of the epithelial channel ENaC, resulting in decreased airway surface liquid volume and further impairment of mucociliary clearance (Butterworth *et al*., 2012). Lipase from *B. cenocepacia* also promotes epithelial invasion by these pathogens (Mullen *et al*., 2010) which may increase their survival rates in the lung.

The unresolved inflammation in the chronically infected patient has a detrimental effect on lung function but may also serve to further enhance pathogen survival in the lung. One recently elucidated mechanism by which this occurs involves the upregulation of the sialyltransferase responsible for modifications to mucins in response to the inflammatory cytokine TNF, resulting in increased levels of the *P. aeruginosa* ligand sLex in bronchial mucosa (Colomb *et al*., 2013). A *B. cenocepacia* strain of the epidemic ET12 lineage activates the TNF receptor 1 (TNFR1) which is likely to play a role in the potent proinflammatory response induced by this species. There is also increasing evidence of a dysregulated TLR4-mediated immune response in CF (Kelly *et al*., 2013) that potentially contributes to the persistently elevated pro-inflammatory mediator levels detected in the lungs of many CF patients. We have also shown that in response to IL-8, *B. cenocepacia* cell growth is enhanced and epithelial invasion levels are increased, another example of enhanced pathogen survival in the presence of a proinflammatory response (Kaza *et al*., 2011). So while many of the pathogen modifications and adaptations serve to avoid the host response and minimize clearance, other strategies are employed which promote inflammation and at least some of these pro-inflammatory responses are beneficial to the colonizing organisms.

Cooperative and competitive interactions between CF pathogens

Given the polymicrobial nature of the CF lung, it is not surprising that CF pathogens and indeed commensals can engage in both intraspecies and interspecies interactions which have the potential to alter the course of airway disease and also pose significant challenges for the management of these patients. The full extent and implications of many of these interactions remains largely unknown although insights into the interactions between the main protagonists in this niche are emerging.

Bacterial–bacterial interactions

Interspecies signalling between *P. aeruginosa* and *Burkholderia* spp. was first reported almost 20 years ago with the demonstration that *B. cenocepacia* exoenzyme secretion is increased in response to *P. aeruginosa* AHLs (McKenney *et al*., 1995). More recent data have further defined some of the features of that interspecies interaction. QS-regulated alginate production by the *P. aeruginosa* mucoid phenotype contributes to *B. cenocepacia* persistence (Chattoraj *et al*., 2010). Furthermore, in a mouse model, co-infection of *P. aeruginosa* and *B. cenocepacia* results in greater levels of biofilm formation and an enhanced inflammatory response (Bragonzi *et al*., 2012).

Interactions between *P. aeruginosa* and *S. aureus* provide a potential explanation for the limited isolation of *S. aureus* in late-stage CF lung disease and the presence of SCVs of *S. aureus* in the patients in which this pathogen persists. *Pseudomonas aeruginosa MucA* mutants which are frequently isolated from chronically infected patients compromise *S. aureus* growth and, unlike wild type *P. aeruginosa*, do not facilitate the formation of *S. aureus* microcolonies (Yang *et al*., 2011). Furthermore, 4-hydroxy-2-heptylquinoline-N-oxide, from the PQS system of *P. aeruginosa*, selects for the highly antibiotic-resistant *S. aureus* SCVs which are also feature in CF microbiology (Hoffman *et al*., 2006). This latter finding is supported by recent clinical data from a French centre indicating that CF patients with methicillin-resistant *Staphylococcus aureus* have poorer outcomes when associated with *P. aeruginosa* infection (Hubert *et al*., 2013).

In addition to the regulation of biofilm formation and alginate production, QS molecules (AHLs) and their degradation products have significant antibacterial activity against both Gram-positive and Gram-negative organisms which may contribute to *P. aeruginosa* dominance in the CF lung. The QS-regulated LasA protein exhibits antistaphylococcal activity (Winstanley and Fothergill, 2009). Tetramic acids, a *P. aeruginosa* AHL degradation product, also exhibits antimicrobial activity against *S. aureus* (Lowery *et al*., 2009) and against Gram-negative bacteria, including the *P. aeruginosa* producer cells (Hosono Honda *et al*., 2011).

In conjunction with the antibacterial characteristics of QS molecules, many bacterial pathogens also produce toxins to target their competitors residing in the same niche. Bacteriocins typically target closely related bacteria, and the inhibition of Bcc by *P. aeruginosa* S-type pyocin (Bakkal *et al*., 2010) is not surprising given the relatedness of these species. Of significance in CF is the fact that the bactericidal activity of *P. aeruginosa* pyocin is greater in anaerobic conditions (Waite and Curtis, 2009), and it is also active against cells in the biofilm mode of growth (Smith *et al*., 2012). Bacteriocin activity by *B. cenocepacia* has also recently been described. Killing by the lectin-like bacteriocin is confined to a limited number of Bcc species and is strain specific (Ghequire *et al*., 2013).

Cooperative pathogen interactions also facilitate the acquisition of antibiotic resistance determinants to more susceptible members of the same species or to different species (El-Halfawy and Valvano, 2013). In this context, polymyxin B-susceptible *P. aeruginosa* appear to benefit from the more resistant *B. cenocepacia* within the same population (El-Halfawy and Valvano, 2013). Dual species biofilms of *P. aeruginosa* with either of two emerging CF species *Inquilinus limosus* and *Dolosigranulum pigrum* resulted in greater tolerance to a range of antibiotics compared with the single species *P. aeruginosa* biofilm (Lopes *et al*., 2012).

Competition for nutrients within the CF microbiome is a seminal factor in pathogen selection, and iron acquisition is a key competitive strategy employed by CF pathogens. The production of siderophores from clinically significant CF pathogens has been extensively reviewed (Mossialos and Amoutzias, 2009; Hammer and Skaar, 2011; Haley and Skaar, 2012); however, the way in which they contribute to shaping the CF microbiome is not fully elucidated. The emergence of siderophore-deficient strains of *P. aeruginosa* that retain the ability to utilize siderophores from other strains is an interesting example of intraspecies cooperation. Furthermore, when in co-culture with *S. aureus*, the frequency of these so-called *P. aeruginosa* cheaters increases, indicative of a competitive interspecies interaction (Harrison *et al*., 2008).

Bacterial–fungal and bacterial–viral interactions

Persistent *A. fumigatus* infection in CF is associated with reduced lung function and increased risk of pulmonary exacerbations. Moreover, there is a significant negative effect on lung function in patients also infected with *P. aeruginosa* (Amin *et al*., 2010). *Pseudomonas aeruginosa* secretory products can inhibit *A. fumigatus* biofilm formation (Mowat *et al*., 2010). The complexity of this particular interkingdom interaction was illustrated using matrix-assisted laser desorption/ionization Time of Flight MALDI-ToF and MALDI-imaging mass spectrometry analysis which revealed suppression, increased production and biotransformation of a range of metabolites. *Aspergillus fumigatus* converts phenazine metabolites of *P. aeruginosa* into compounds with enhanced toxicities and which can induce fungal siderophores (Moree *et al*., 2012). The interactions between *Candida albicans* and *P. aeruginosa* have been more extensively studied. *Pseudomonas aeruginosa* secretory products including the pyocyanin inhibit the growth of *C. albicans* and *P. aeruginosa* binds to the hyphal surfaces of the fungus (Hogan and Kolter, 2002). However, *C. albicans* transition to the hyphal form is inhibited by a homoserine lactone QS molecule secreted by *P. aeruginosa*, thereby protecting the fungus from bacterial attack (Hogan *et al*., 2004). Furthermore, *C. albicans* secretes a QS molecule, farnesol which inhibits the secretion of the QS signal PQS by *P. aeruginosa* (Cugini *et al*., 2007). Interestingly, a *B. cenocepacia* QS signal, *cis*-2-dodecenoic acid, also inhibits the morphological transition in *C. albicans* (Boon *et al*., 2008), further evidence of significant interkingdom interactions that potentially shape the microbial landscape in CF.

The specific role of viral infection in CF lung disease is poorly defined, although it is widely accepted that viruses do cause airway disease exacerbations. One potential mechanism by which viruses contribute to a decline in lung function is the enhanced binding of *P. aeruginosa* to epithelial cells facilitated by respiratory syncytial virus (Van Ewijk *et al*., 2007). A recent study has shed light on the complexity of the viral contribution by showing that a different profile of host response genes are activated by rhinovirus compared with influenza virus infection in CF (Ramirez *et al*., 2014). Given the high rhinoviral load in the lungs of children with CF (Kieninger *et al*., 2013), further investigations of the role of viruses, their effects on the lung microbiome and their direct or indirect impact on lung function are clearly warranted.

Studying bacterial interactions

Understanding interspecies interactions and bacterial–host interactions are the key to effective management and elimination of lung pathogens and to date a range of model

systems including *in-vivo* insect, animal and fish models, and *in-vitro* cell culture systems have all contributed to our knowledge of these interactions. Sibley and colleagues (2008b) developed a *Drosophila melanogaster* model in which microbe–microbe interactions of *P. aeruginosa* isolates with consequential modulation of virulence factor expression have been examined. New *in vitro* techniques are being developed to study complex polymicrobial infections. Connell and colleagues (2013) have devized a microscopic three-dimensional (3D) printing strategy that enables multiple populations of bacteria to be organized in various 3D geometries, including adjacent, nested and free-floating colonies. In this laser-based lithographic technique, microscopic containers are formed around selected bacteria suspended in gelatin via focal cross-linking of polypeptide molecules. Microfabrication technology has been successfully applied to the study of QS signalling responses of single *S. aureus* cells encapsulated in microdroplets of lipid-silica (Carnes *et al*., 2010) and to the signalling involved in *P. aeruginosa* biofilm formation using an array of mini-culture chambers formed with permeable PEG (polyethylene glycol) diacrylate hydrogel (Flickinger *et al*., 2011). These novel technologies coupled with high-throughput genomic and proteomics are beginning to define and characterize the complex relationships within microbial communities including that of the CF lung.

Concluding remarks

As the CF community awaits the development of additional CFTR modulators targeting the range of CFTR mutation classes or the development of an effective gene replacement therapy, the management of respiratory infections in this complex niche remains the primary focus. Progress has been made in terms of defining the diversity of the microbial community in the CF lung and in understanding how some of these bacterial species interact with each other and with the host. The significance of bacterial load has been established for particular pathogens which should begin to inform therapeutic intervention. The important role of synergistic interactions between pathogens is also emerging and will bring into focus species that may not be inherently virulent but make an indirect contribution to disease progression. Therefore, the challenge now for scientists and clinicians alike is to translate emerging microbiome data into clinical interventions that will significantly improve patient outcomes.

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