

## Mini-Review

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## Microbial ecology of the cystic fibrosis lung

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Understanding the microbial flora of the cystic fibrosis (CF) respiratory tract is of considerable importance, as patient morbidity and death are primarily caused by chronic respiratory infections. However, chronically colonized CF airways represent a surprisingly complex and diverse ecosystem. The precise contributions of different microbes to patient morbidity, and in particular the importance of inter-specific interactions, remain largely unelucidated. The importance of within-species genetic and phenotypic variation has similarly received limited explicit attention. While a host of studies provide data on the microbial species recovered from patients, these are often incomparable due to differences in sampling and data reporting, or do not present the data in a way that aids our understanding of the ecosystem within each patient. This review brings together a cross-section of recent research on the CF airways and the microbes which infect them. The results presented suggest that understanding the CF lung in terms of its community and evolutionary ecology could benefit our understanding of disease progression and influence treatment regimens.

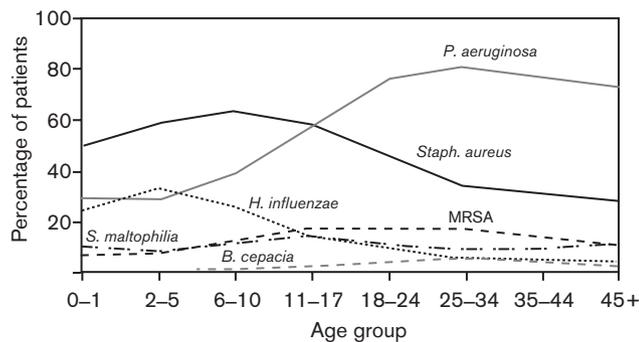
## Introduction: cystic fibrosis lung communities

The airways of cystic fibrosis (CF) patients are colonized by pathogenic micro-organisms in infancy, and in the vast majority of cases chronic infections are established. Most patients experience recurrent acute respiratory episodes and eventually die of respiratory failure resulting from infection (Lyczak *et al.*, 2002). Most studies of CF pathogens have focussed on four major bacterial species: *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex (BCC). The prevalence of these species in the airways changes over time, as illustrated in Fig. 1. Most pathogens are acquired from environmental reservoirs, but patient-to-patient transmission also occurs (LiPuma *et al.*, 1990; Salunkhe *et al.*, 2005). The microbiology of the airways can therefore also be influenced by contact between patients (and thus treatment centre isolation policies, attendance of patient support groups, etc.). Colonization by *P. aeruginosa* severely worsens prognosis, promoting more frequent acute exacerbations and a general decline in lung function (Alvarez *et al.*, 2004; Nixon *et al.*, 2001).

Numerous other bacteria invade the CF airways and contribute to morbidity, including *Stenotrophomonas maltophilia* (shown in Fig. 1), *Alcaligenes xylosoxidans* and *Klebsiella* spp. (Lambiase *et al.*, 2006; Lyczak *et al.*, 2002). The range of species reported has widened over the past few decades as life expectancy has increased and detection methods have improved (Rogers *et al.*, 2004). Pathogenic viruses [e.g. respiratory syncytial virus (RSV), adenoviruses, influenza: van Ewijk *et al.*, 2005] and fungi (e.g. *Aspergillus* and *Candida* species: Haase *et al.*, 1991) are also common.

In recent years, some CF centres have reported that early, aggressive antibiotic treatment or prophylactic administration of antibiotics can delay or prevent chronic colonization by *P. aeruginosa* and *Staph. aureus* (Döring & Hoiby, 2004; Hoiby *et al.*, 2005; Lebecque *et al.*, 2006). While promising, these initial results are generally based on small samples and treatment does not prevent chronic colonization in all patients. Given the prevalence of CF, small failure rates correspond to a large number of patients for whom alternative therapies must be employed. Further, patients who avoided chronic colonization in these studies still experienced intermittent colonization by the target bacterium, and the incidence of infections by other species was not quantified. Thus the hope of early, aggressive intervention becoming the norm should not lead us to stop trying to understand what happens in the airways when patients do become colonized – especially given the risk of emergent antibiotic resistance.

There exists a wealth of studies of CF lung microflora (see Fig. 2). Unfortunately, our ability to spot patterns in the available data – particularly patterns of coinfection by different species – is hampered by the fact that many surveys do not explicitly report coinfections on a per-patient basis (notable exceptions being Moore *et al.*, 2005; Rogers *et al.*, 2004), do not state patient genotype or disease phenotype (e.g. Burns *et al.*, 1998; Santana *et al.*, 2003), do not record previous antibiotic treatment (e.g. Alvarez *et al.*, 2004) and/or pool samples from different parts of the respiratory tract (e.g. Nixon *et al.*, 2001). Different surveys use different inclusion criteria and focus on different groups of microbes, with most studies reporting only bacterial pathogens. The



**Fig. 1.** Prevalence of bacterial respiratory infections by age group in CF subjects. [Reproduced, with modifications, with permission from the Cystic Fibrosis Foundation National Patient Registry (Cystic Fibrosis Foundation, 2004).] MRSA, methicillin-resistant *Staph. aureus*.

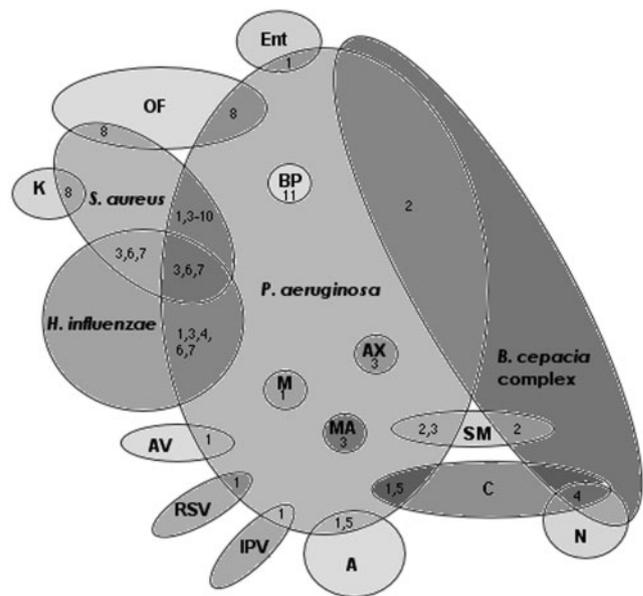
picture we have of the lung communities present in CF patients is, therefore, limited and qualitative.

There are, however, three broad conclusions to be drawn from the published literature. First, coinfections involving different species of bacteria, or bacteria, fungi and viruses, are common and probably the norm. Second, coinfecting species interact, both synergistically and antagonistically. Finally, pathogen populations within the lung evolve in response to selection pressures exerted by the within-host environment and by other members of the community. These points will now be discussed.

### Coinfections and communities

Several studies explicitly report coinfections of pairs or triplets of bacterial species, while in other cases we can infer from the data given for individual species that a significant proportion of patients were infected by more than one species. Anzaudo *et al.* (2005) reported that 31 % of patients harboured two or more bacterial species simultaneously, while Hoiby (1974) and Wahab *et al.* (2004) reported coinfections in 'most patients'. Burns *et al.* (1998) calculated that they recovered an average of 2.9 species of pathogenic bacteria per sample (range: 0–10), and more recent DNA profiling studies suggest that CF bacterial communities may be even more diverse than suggested by culture-based methods (Rogers *et al.*, 2004). Further, Petersen *et al.* (1981) found that 13 % of acute respiratory exacerbations among their study population were associated with simultaneous bacterial and non-bacterial infections. The results of a review of reported coinfections are summarized in Fig. 2.

We have, then, evidence for high inter-species diversity in the CF lung. Significant intra-species diversity is also observed. Burns *et al.* (1998) recovered, on average, 2.38 phenotypically distinct *P. aeruginosa* isolates per sample per patient (range: 1–6). Further, selection pressures on pathogens are likely to be both temporally and spatially



**Fig. 2.** Venn diagram showing reported coinfections of the CF airways. (NB: coinfection does not necessarily imply direct interaction between species.) A, *Aspergillus* spp.; AV, adenovirus; AX, *A. xylosoxidans*; BP, bacteriophage; C, *Candida* spp.; Ent, enterobacteria; IPV, influenza and/or parainfluenza virus; K, *Klebsiella* spp.; M, mycoplasma; MA, *Mycobacterium abscessus*; N, *Neisseria* spp.; OF, oropharyngeal flora; RSV, respiratory syncytial virus; SM, *S. maltophilia*. Numbers refer to references: 1, Petersen *et al.* (1981); 2, Lambiase *et al.* (2006); 3, Wahab *et al.* (2004); 4, Moore *et al.* (2005); 5, Burns *et al.* (1998); 6, Hoiby (1974); 7, Lording *et al.* (2006); 8, Santana *et al.* (2003); 9, Alvarez *et al.* (2004); 10, Anzaudo *et al.* (2005); 11, Ojeniyi *et al.* (1991).

heterogeneous during long-term infections, leading to genetic diversification of founder clones. Smith *et al.* (2006) genotyped *P. aeruginosa* isolates taken from a single CF patient over a period of 90 months and showed the existence of multiple, related lineages which coexisted for long periods.

Different microbial communities can be found within the same patient. Several authors have noted a poor correlation between species and/or strains present in upper and lower airway communities (Armstrong *et al.*, 1996; Lyczak *et al.*, 2002; Saiman, 2004) and in different lobes of the lung (Gutierrez *et al.*, 2001; Smith *et al.*, 1998). This is unsurprising given the compartmentalized nature of the respiratory system and high viscosity of CF mucus, and means that pooling samples from different lobes (as in Nixon *et al.*, 2001) is unlikely to give a reliable picture of the microbial ecology within a patient.

The outcome of a mixed (multi-strain, multi-genotype or multi-species) infection depends on exactly how coinfecting populations interact. Clinical and laboratory studies have started to elucidate the many ways in which CF pathogens might interact *in vivo*.

## Community interactions 1: synergism

Coinfections probably result from sequential, rather than simultaneous, colonization, and it appears that some degree of succession (change in community structure over time) occurs within the CF lung (e.g. Hoiby, 1974; Wahab *et al.*, 2004). Molecular profiling studies such as that of Rogers *et al.* (2004) provide particularly clear evidence for succession within patients. Succession may depend in part on environmental changes resulting from the action of infecting microbes; alginate production by *P. aeruginosa*, for example, can exacerbate local hypoxia (Worlitzsch *et al.*, 2002), presumably constraining the spectrum of species or strains able to invade. There is also evidence that RSV may render some patients more susceptible to chronic *P. aeruginosa* infection (Petersen *et al.*, 1981).

Historically, synergism in the CF lung has usually been proposed with respect to *Staph. aureus* 'sensitizing' the lungs to subsequent infection by *P. aeruginosa* (e.g. Burns *et al.*, 1998; but see also Lyczak *et al.*, 2002). However, there is also evidence for antagonistic interactions between these two species (see below). On the other hand, there is very good evidence that *P. aeruginosa* can promote further pathogenesis. Firstly, two endobronchially detectable *P. aeruginosa* quorum-sensing molecules (3-oxo-C<sub>12</sub>-homoserine lactone and 2-heptyl-3-hydroxy-4(1H)-quinolone) have been shown to possess immunomodulatory effects (Hooi *et al.*, 2004). Secondly, Wahab *et al.* (2004) reported a possible link between pre-existing, chronic colonization by mucoid *P. aeruginosa* and infection by more rarely observed bacteria such as *S. maltophilia*, *A. xylosoxidans* and *Mycobacterium abscessus*. Moreover, *P. aeruginosa* has been implicated in promoting BCC pathogenesis via the upregulation of BCC virulence factor expression (Riedel *et al.*, 2001).

Synergism can result in increased disease severity in mixed-species infections. For instance, those patients with coinfections of mucoid *P. aeruginosa* and rare bacterial species observed by Wahab *et al.* (2004) had significantly worse lung symptoms than did patients colonized by mucoid *P. aeruginosa* alone. The normal oropharyngeal microflora (OF) may similarly exacerbate CF lung disease. In CF patients, avirulent species which are normally confined to the upper airways are frequently recovered from the lower airways (Coenye *et al.*, 2002; Santana *et al.*, 2003). It has been shown (Duan *et al.*, 2003) that the presence of OF exacerbates *P. aeruginosa*-mediated lung damage in infected rats. This resulted from the specific upregulation of *P. aeruginosa* genes linked with pathogenesis, and appeared to be partially attributable to the *P. aeruginosa* cells 'eavesdropping' on intercellular communication between OF constituents (Duan *et al.*, 2003).

Bacteriophage are present at appreciable concentrations in the CF airways (Ojeniyi *et al.*, 1991), and these may affect the pathology of their target bacteria. *P. aeruginosa* phage recovered from CF sputum have been shown to be capable of inducing changes in *P. aeruginosa* serotype and a shift to

polyagglutinability – a trait associated with increased patient mortality (Hoiby & Rosendal, 1980).

## Community interactions 2: antagonism

Antagonism between organisms within a community is unavoidable due to competition for finite resources. This may involve simple resource competition or direct antagonistic effects on the growth or viability of competitors (interference competition).

There is particularly good evidence for antagonism between *P. aeruginosa* and *Staph. aureus*. *P. aeruginosa* can lyse the cells of *Staph. aureus* and other Gram-positive bacteria (Mashburn *et al.*, 2005; Palmer *et al.*, 2005) and it has been demonstrated that CF sputum induces this behaviour, increasing the competitive ability of *P. aeruginosa in vitro* (Palmer *et al.*, 2005). In the case of *Staph. aureus*, *P. aeruginosa* can use the iron released by lysis to support its own growth (Mashburn *et al.*, 2005), but the relevance of this within the CF airways is not known.

Two further types of antagonistic interaction have received much attention in the literature on microbial evolution and ecology, but their possible roles in the CF lung have not been explicitly addressed. These are bacteriocin production and the breakdown of cooperative behaviours. Bacteriocins are molecules produced by bacteria which kill cells belonging to another clone (Michel-Briand & Baysse, 2002). Their action is usually limited to conspecific clones, although some bacteriocins play a role in interspecific competition (Michel-Briand & Baysse, 2002). There is a growing body of work on the ecology of bacteriocin production, and it has been shown that bacteriocin-mediated competition can affect virulence. When cells invest resources in bacteriocin production, they reduce their own growth rate as well as that of any sensitive competitors. The result is a reduction in total bacterial density, and hence virulence, in mixed infections (Massey *et al.*, 2004). Bacteriocin production is more common in clinical isolates than in environmental strains (Govan, 1986; Chang *et al.*, 2005), but the extent to which it influences CF lung community structure and virulence has not been addressed. As some authors have suggested therapies based on bacteriocins (e.g. (Michel-Briand & Baysse, 2002) it may be worthwhile examining the relevance of this behaviour in the CF lung in more detail.

Another type of antagonism is the breakdown of cooperative behaviours due to the evolution of non-cooperative 'cheats'. Microbial populations often increase their growth rate by acting cooperatively – generally by producing 'public goods' such as iron-scavenging siderophores, toxins and degradative enzymes (West *et al.*, 2006). Within a cooperating population, there exists an advantage to becoming a non-cooperating 'cheat'; i.e. investing nothing in public good production but utilizing the molecules produced by one's neighbours. If cheats reach sufficiently high frequencies, the effect on resource exploitation and so population density will be significant (Harrison & Buckling, 2005). Some public

goods are directly necessary for pathogen virulence, and in such cases cheat-mediated antagonism can decrease virulence (Harrison *et al.*, 2006). Siderophore production by *P. aeruginosa* is a classic example of cooperation (Griffin *et al.*, 2004). However, while necessary in acute infections (Harrison *et al.*, 2006), siderophore production decreases over time in chronic infections (De Vos *et al.*, 2001; Smith *et al.*, 2006). Whether this decline is due to a decreased need for siderophores as more iron becomes available in the CF lung (Stites *et al.*, 1999; but see also Haas *et al.*, 1991), or as a result of increased local competition and/or decreased relatedness (Griffin *et al.*, 2004), remains to be seen. Furthermore, patterns of siderophore production over time can differ markedly between patients (De Vos *et al.*, 2001). If selection pressures differ between patients, it would be useful to determine whether this is attributable to differences in microbial community structure, to differences in the within-host environment, or to founder effects of the colonizing clone. Unfortunately we cannot answer this question with the data currently available. Moreover, the nature of any relationship between siderophore production and CF disease phenotype has not been addressed.

The presence of micro-organisms elsewhere in the host appears to affect the immune response to respiratory infection. In particular, there is evidence that maintaining a healthy gut microflora can antagonize the success of respiratory pathogens. In mice, dietary supplementation with *Lactobacillus casei* or natural yoghurt has been shown to stimulate the activity of alveolar macrophages, increase clearance of respiratory *P. aeruginosa* (Alvarez *et al.*, 2001) and expedite recovery from subsequent *Streptococcus pneumoniae* infection (Villena *et al.*, 2005).

### Evolution within the lung

During chronic infection, pathogens will experience changing selection pressures as they encounter new habitats and different coinfecting species and as they respond to medical intervention. Most of the work on pathogen evolution in the CF lung has focussed on *P. aeruginosa*. Smith *et al.* (2006) found evidence of widespread positive selection across the *P. aeruginosa* genome during 8 years of infection in one patient. The ratio of nonsynonymous to synonymous base changes per site was calculated to be 1.4 and around two-thirds of the nonsynonymous mutations were predicted to alter protein function. These authors also reported the *in vivo* evolution of divergent lineages with different combinations of mutations (mainly single base pair mutations or 1–3 base pair indels). Kresse *et al.* (2003) also reported adaptation and diversification of this species within the lung, with some novel genotypes enjoying a long-term selective advantage. These authors also found numerous large-scale rearrangements and indels in evolved genotypes.

The most commonly observed phenotypic changes in CF bacterial pathogens over time are antibiotic resistance (Anzaudo *et al.*, 2005; Burns *et al.*, 1998; Lambiase *et al.*,

2006; Lyczak *et al.*, 2002), mucoidy (Lyczak *et al.*, 2002; Nixon *et al.*, 2001), a loss of cell motility (Mahenthalingam *et al.*, 1994), the appearance of ‘small colony variants’ (Haussler *et al.*, 2003; Besier *et al.*, 2007), increased mutation rate (Ciofu *et al.*, 2005) and decreased production of virulence factors associated with acute infections. This last is thought to aid in immune evasion (Smith *et al.*, 2006). The results of Smith *et al.* (2006) are consistent with these observations, as they recovered numerous loss-of-function (nonsense or frame shift) mutations in genes involved in twitching motility, biofilm formation and the production of compounds required for the establishment of acute infections, as well as mutations that produced a hypermutable phenotype and numerous mutations in multidrug efflux pumps. Efflux pump mutants were shown to have increased resistance to a range of antibiotics.

Some of these evolved traits may be linked with community interactions. For instance, the presence of pilus-binding bacteriophage may select against expression of type IV pili by *P. aeruginosa* and so lead to a loss of twitching motility and biofilm formation.

The increases in both inter- and intra-specific microbial diversity observed during chronic infection (De Vos *et al.*, 2001; Kresse *et al.*, 2003; Smith *et al.*, 2006) could have several possible effects on social interactions. On the one hand, decreases in relatedness could reduce virulence as a result of decreased cooperative host exploitation, or if interference competition maintains a lower total population density. On the other hand, low relatedness could increase virulence if scramble competition favours more voracious clones. The effect of relatedness on virulence has received much discussion, and it has become clear that this relationship depends on the nature of the interactions between coinfecting clones and on the exact behaviours required for host exploitation (West *et al.*, 2006).

### How can ecology inform therapy?

The CF airways represent an evolving ecosystem, with interactions between pathogens affecting disease phenotype. It is therefore advisable to evaluate prophylactic intervention in terms of its community-level effects. For instance, antibiotic treatment to remove particular species will have limited success if other members of the community predispose the patient to recolonization, if treatment simply creates selection pressure for resistance, or if removal of the target pathogen creates an opportunity for other pathogen populations to invade or expand. Additionally, not understanding how populations grow *in vivo* can mean that *in vitro* tests of potential therapies produce unreliable results. If we can improve our picture of pathogen ecology within the lung, this will facilitate the development of more promising therapies.

Certain authors (e.g. Moore *et al.*, 2005) have suggested that artificial manipulations of lung communities could form the basis for more effective treatment of chronic infections.

Treatments based on molecules which block bacterial quorum sensing (Eberl, 2006; Le Berre *et al.*, 2006), bacteriocins (Michel-Briand & Baysse, 2002), bacteriophage (Toro *et al.*, 2005) and bacteriovirus vibrios (Kadouri & O'Toole, 2005) have all been suggested. Such interventions could form a prelude to more conventional treatments and increase their efficacy. Some trials of 'ecological medicine' have shown promising results (e.g. Toro *et al.*, 2005). It is clear, however, that a better understanding of the microbial ecology of infections is not only important for predicting their virulence, but is also necessary if we are to develop reliable and safe methods of disrupting these communities. For instance, high doses of pilus-dependent lytic phage administered during the early stages of infection may reduce pathogen population size, but given the potential for some phage to enhance bacterial adaptation and persistence, the details of how specific phage interact with their target bacteria must be elucidated before potentially useful phage can be identified.

Therapies that directly target the airways may not be the only means of increasing resilience to infection. The discovery that a healthy gut microflora protects against respiratory pathogens may prove to be a valuable one. It is especially pertinent as many CF patients receive oral or intravenous antibiotics, whose actions are not localized to the respiratory tract and which could deplete gut microflora. Dietary supplementation with probiotics or prebiotics and/or the preferential use of inhaled antibiotics may be sometimes be advisable and could bolster the success of more direct interventions.

### Conclusions: integrating ecology, evolution and medicine

The microflora of the CF airways commonly consists of numerous multi-species assemblages. Interactions between clones and between species are likely to determine morbidity. Over time, with repeated medical intervention and new colonization events, these communities evolve and their characteristics can change significantly. Our understanding of this ecosystem is sketchy: while we have identified the major pathogenic species and mutants, our knowledge of how these interact within the community is patchy. Understanding the evolutionary ecology of CF airway infections could help in the search for novel or improved treatments. As it is, the successful removal of one pathogen strain from the lung simply leaves an empty niche which can often rapidly be filled by a new invader (see e.g. Hoiby & Rosendal, 1980). Given the promising results of early eradication trials, it would also be useful to ascertain how the microbial communities differ in intermittent versus chronic infections. If host factors that affect characteristics of the pathogen community and the course of lung pathogenesis could be identified, there is a chance that future treatments could be optimized to the needs of particular patients.

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