Adaptation and Evolution of Pathogens in the Cystic Fibrosis Lung

Paul J. Planet[1,2,3]

[1]Division of Pediatric Infectious Diseases, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA, [2]Department of Pediatrics, Perelman College of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA, and [3]American Museum of Natural History, New York, New York 10024, USA

As opposed to acute respiratory infections, the persistent bacterial infections of the lung that characterize cystic fibrosis (CF) provide ample time for bacteria to evolve and adapt. The process of adaptation is recorded in mutations that accumulate over time in the genomes of the infecting bacteria. Some of these mutations lead to obvious phenotypic differences such as antibiotic resistance or the well-known mucoid phenotype of *Pseudomonas aeruginosa*. Other mutations may be just as important but harder to detect such as increased mutation rates, cell surface changes, and shifts in metabolism and nutrient acquisition. Remarkably, many of the adaptations occur again and again in different patients, signaling that bacteria are adapting to solve specific challenges in the CF respiratory tract. This parallel evolution even extends across distinct bacterial species. This review addresses the bacterial systems that are known to change in long-term CF infections with a special emphasis on cross-species comparisons. Consideration is given to how adaptation may impact health in CF, and the possible evolutionary mechanisms that lead to the repeated parallel adaptations.

Key words: adaptation; cystic fibrosis; in host evolution; microbiota.

In the 1960s, Robert Doggett and colleagues [1] first described a strong association of unusual *Pseudomonas aeruginosa* isolates that produced copious amounts of a mucoid material with cystic fibrosis. The association was striking because *P. aeruginosa* was considered to be only rarely encapsulated [2]. This material was later identified as alginate [3, 4], a polysaccharide originally isolated from brown seaweeds [5]. In addition, there seemed to be a clear progression, with nonmucoid *P. aeruginosa* isolated in early disease and mucoid strains appearing later, sometimes present alongside nonmucoid strains and sometimes on their own. The crucial question was whether this observed "change" was due to the introduction of mucoid *P. aeruginosa* replacing or outcompeting existing nonmucoid strains, or whether it was possible that the infecting strains had gained, newly, the capacity to make alginate. The paucity of mucoid environmental strains led initially to the suspicion that strains were converting, rather than being replaced, which was confirmed with the advent of molecular typing techniques [2].

The finding that many mucoid strains were highly similar to nonmucoid strains from the perspective of sequence type, suggested that this was evidence of "intra–host" evolution, and the repeated (parallel) evolution in multiple patients argued that there were strong selective pressures at work that made mucoidy highly beneficial to the infecting organisms. The fitness benefits proposed focus on the physical barrier provided by alginate, with protection from immune cells such as neutrophils and alveolar macrophages, reduced penetration of antibiotics, and immune molecules such as complement and immunoglobulins [2, 6]. Adherence and production of a difficult to remove biofilm also may be important for persistence [7].

That a major phenotypic change could occur in *P. aeruginosa* opened up the possibility that other pathogens were adapting genetically to the CF niche, and that other phenotypic changes might be occurring. The recent development of whole genome sequencing as a major tool for understanding long-term infection in CF has helped to clarify the major patterns of genomic and phenotypic evolution, in addition to identifying new types of adaptations that had gone unnoticed before [8–10]. The critical quality of in-host evolution is that it occurs recurrently in multiple individuals with the same disease and even within the same patient in distinct infecting lineages, providing the strongest evidence we have that these specific changes are beneficial to infecting organisms [10–14].

WHAT IS THE CF RESPIRATORY TRACT LIKE?

To understand bacterial adaptation to the CF airway, it is important to understand what the niche is like from the bacterial perspective, and therefore what fitness obstacles need to be overcome. First, the CF respiratory niche is probably best

described as multiple niches that extend from the upper airway all the way through to the deepest alveolae, with multiple distinct environments and atmospheres. There are areas of high oxygen with nearby areas that, after a steep oxygen gradient, are effectively anaerobic. In this milieu, there is a lack of critical nutrients such as iron and zinc, and areas with high concentrations of oxygen radicals [15], varying levels of antibiotics, and immune molecules and cells. There are also other organisms replicating and occupying this space that compete for some of the same nutrients, and that may directly attack with bacteriocins or other toxic molecules. Areas of the lung with different amounts of mucous plugging, bronchiectasis, and aeration may present very distinct niches. The heterogeneity of the CF airway, the relative lack of movement between spaces, and the failure to clear bacteria quickly sets up the perfect environment for hundreds of local evolutionary experiments to play out. Indeed, multiple studies have shown phenotypic and genomic diversity in distinct parts of the lung upon explant [16–18].

ANTIBIOTIC RESISTANCE AND TOLERANCE

Antibiotic resistance is the most obvious clinical problem that arises from intra-host evolution. CF patients have very high levels of antibiotic exposure, which selects for pathogens that can evade antibiotic toxicity. Indeed, one reason that *P. aeruginosa* is a common pathogen in CF is likely because of its inherent antibiotic resistance to multiple drugs. Likewise, multidrug resistant (MDR) organisms are favored under standard CF treatment regimens, which not only consist of periodic treatments with broad spectrum antibiotics for pulmonary exacerbations but also long-term use of aminoglycosides (e.g., inhaled tobramycin and amikacin), monobactams (e.g., inhaled aztreonam), and oral azithromycin [19–21].

High exposure to antibiotics does not just lead to invasion of MDR pathogens. It can also lead to mutations in existing populations true antibiotic resistance or tolerance. Antibiotic tolerance is defined here as either an increased, but intermediate, minimal inhibitory concentration (MIC) or the ability to survive antibiotic challenge. The mechanisms of antibiotic resistance are as diverse as the mechanisms of resistance themselves. Mutations in genes for multidrug efflux pumps and associated proteins have been noted in *P. aeruginosa*, *Burkholderia* species, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans* [9, 12, 22–24]. These mutations often result in changes in MIC to multiple antibiotics at the same time. Mutations in porin genes, which diminish the diffusion of antibiotics into the bacterial cell, have been described for *P. aeruginosa* [9] and *Burkholderia* spp. [23]. In addition, point mutations for aminoglycoside, macrolide, and rifampin resistance have been described for nontuberculous mycobacteria (NTM) [25]. Point mutations in *ampD*, whose protein product controls the beta-lactamase AmpC expression, have

been described for *B. multivorans* [23]. For *Staphylococcus aureus*, point mutations for linezolid and azithromycin have been described [26].

Several other adaptations seem to affect the ability of bacteria to weather an antibiotic challenge. Adaptations that enhance biofilm formation are the most recognized form of antibiotic tolerance in that they provide a barrier to diffusion of antibiotics [27, 28]. Biofilms also harbor distinct populations of cells, some of which may be less metabolically active and less susceptible since antibiotics preferentially kill metabolically active or dividing cells [29]. In general, bacteria with a less active metabolic state may be more tolerant to antibiotics, and this has led to the study of small colony variants (SCVs), metabolic auxotrophs, and, so-called persister cells in several organisms including *S. aureus* and *P. aeruginosa* [30–33].

EXTRACELLULAR APPENDAGES AND CELL ENVELOPE CHANGES

Bacteria present multiple molecules and appendages on their surface that can trigger an immune and inflammatory response [34], but these same surface factors (pili, flagella, LPS) are critical for bacterial motility, colonization, and interaction with their surroundings. Therefore, while they enable infection [35], they are also a liability for pathogens once they have established a stable infection. For *P. aeruginosa*, a recurrent and predictable change associated with adaptation to the host is the loss of flagella [9, 36], a major stimulator of the Toll-like receptor 5 (TLR5) [37], and type IV pili [9, 38, 39], for which an innate immune response is not well characterized. Loss of motility, due to the loss of flagella [40] has also been observed in *Burkholderia* spp., along with reduced TLR5 stimulation for nonmotile strains, although only a minority of the observed strains underwent this transition [41].

Changes in the surface exposed cell envelope is most well-known for lipopolysaccharide (LPS) in the outer membrane of gram-negative bacteria. *P. aeruginosa* strains have been observed that change the molecular conformation of their LPS, becoming less immunostimulatory [42–46]. Strains of *P. aeruginosa* isolated from CF lungs often do not express high molecular weight O antigen (displayed on LPS) which may make these strains more susceptible to killing in human serum [47, 48]. Loss of the LPS O antigen has also been noted to occur in *B. multivorans* infection through mutations in the *wbi* gene cluster [12]. Larger surveys have suggested that rates of loss of O antigen may be specific for each *Burkholderia* species with *B. cenocepacia* and *B. multivorans* having particularly high rates of conversion [49]. O antigen loss may lead to lower immunogenicity and evasion of the host immune response [50], or potential increased persistence through increased intracellular survival [51].

For gram-positive organisms such as *S. aureus*, innate immune responses linked to sensing by TLR2 of peptidoglycan

might be expected, but no such adaptive changes have been described. However, for nontuberculous mycobacteria (NTM) there have been changes noted in the glycopeptidolipids (GPL) of the outer cell envelope that lead to changes in immunostimulation as well as other phenotypic properties such as rough and smooth colony morphologies, biofilm formation, aggregation, and survival of macrophage killing [52].

While the pervasive changes in the superficial or exposed aspects of a bacterial cell in the CF lung are likely to be tightly linked to the immune response, it is worthwhile considering other possible advantages that losses or changes in these factors may have for bacteria. Indeed, some changes associated with LPS may arise simply because of selection for biofilms and CF-like nutritional regimes [53, 54]. Also, once established in a thriving community in the lungs, bacteria may no longer need to expend the energy for appendages that are primarily advantageous for colonization and motility. Other adaptations such as deep embedding in an alginate matrix may make these activities much less relevant. In general, reductive or degradative genomic evolution appears to be a theme for CF pathogens (see below). In this type of evolution, especially in bacteria with higher mutation rates or more active transposition or recombination, genes that are not needed are easily lost. It seems possible that many of the losses observed and taken as signs of adaptation are merely the consequence of random inactivation of unneeded systems.

MUCOIDY, BIOFILM FORMATION, AND QUORUM SENSING

As noted above, mucoidy in *P. aeruginosa* was the first attribute that appeared to arise in a highly parallel fashion in CF patients. The mechanism of the transition to mucoidy has been deeply studied, and has been shown to originate from derepression of regulatory genes of alginate production, particularly *algU, algW, mucA, mucB, mucD* [55, 56]. These mutations have been seen repeatedly in multiple recent whole genome studies with loss of function mutations arising in multiple genes involved in the regulation of the production of the exopolysaccharide (EPS) alginate [14]. Organisms grown in an alginate biofilm are less susceptible than planktonic cells to antibiotic challenge and more protected from the immune system response [28, 57, 58].

While *B. cepacia* complex isolates have been shown to form biofilms [59–61], mucoid isolates were previously thought to be rare, especially in the environment [2]. However, some CF centers have reported that more than 80% of isolates from CF patients make EPS [62, 63], and culture conditions also greatly impact EPS production with environmental strains also producing EPS [64–66]. The most widespread *B. cepacia* EPS, cepacian [67, 68], is involved in the thickness and maturity of biofilms in some strains but not all [63]. Murine models show increased persistence of mucoid isolates compared to isogenic

controls [69], and EPS has also been implicated in scavenging oxygen radicals and interfering with neutrophil chemotaxis [70]. However, the mucoid phenotype is complex, and in surveys from CF patients, *Burkholderia* species have highly variable biofilm-forming capacity, colony morphology, and levels of EPS production [63, 64]. Thus, while the impact of EPS remains unclear the virulent species *B. cenocepacia* has a high frequency of EPS producing isolates [64]. However, one study showed that patients with nonmucoid strains had more rapid lung function decline and worse outcomes [71]. Interestingly, unlike the pattern of change in *P. aeruginosa*, longitudinal *Burkholderia* isolates appear to switch both from mucoid to nonmucoid and vice versa, with the majority of phenotypic changes occurring from mucoid to nonmucoid [64].

Biofilms can take different forms and have distinct extracellular matrix components including exopolysaccharides other than alginate, and other important components such as pili or DNA [7, 72–74]. Another phenotypic switch noted for persistent *P. aeruginosa* infections in CF is the transition to rugose small colony variants (RSCVs), which is tied to the expression of nonalginate exopolysaccharides Psl and Pel [75–77]. RSCVs of *P. aeruginosa* form strong attachments to surfaces and pneumocytes, autoaggregate, and have been found in significant quantities in CF patients [78, 79]. Some are hyperpiliated and have increased twitching motility [78]. RSCVs have also been shown to be more resistant to phagocytosis [80, 81]. Multiple other phenotypes have been associated with *P. aeruginosa* RSCVs including both increases and decreases in cytotoxicity and virulence [31]. The original RSCV phenotype was described as the result of overproduction of cyclic–diGMP and linked to the genes *pvrR*, encoding a cyclic-di-GMP phosphodiesterase, and *wspR*, encoding a diguanylate cyclase [82, 83]. Multiple additional naturally occurring mutations have been linked to the RSCV phenotype, the most common are loss-of-function mutations in genes whose products negatively regulate cyclic-di-GMP production (e.g., *wspF, fleQ, yfiN, yfiR*) [9, 80, 81]. The second messenger cyclic-di-GMP is involved in the control of multiple processes that are central to the adaptive processes of *P. aeruginosa* to the CF environment including elaboration of extracellular appendages, exopolysaccharide, adhesins, virulence and cytotoxicity systems among others [31]. Interestingly, recent work ties the mutations that lead to loss of flagella to Pel and Psl production, suggesting that the loss flagella may just be a "side effect" of the true fitness benefit of creating a Psl/Pel biofilm [75]. *Burkholderia cenocepacia* has also been noted to easily produce RSCVs in in vitro evolution studies that are due to *wsp* locus genes [84], and there are reports of cystic fibrosis patients with the SCV phenotype [85].

Bacteria living in close proximity are well known to communicate with each other through small molecules generally known as quorum sensing. In long-term infections in CF, *P. aeruginosa* has been noted to lose quorum sensing abilities

through mutation of *lasR* and *rhl* genes [38, 86–89]. The loss of quorum sensing has multiple impacts including modulation of expression of many other genes including those with an impact on virulence [90, 91]. It also may lead to increased growth rates due to more efficient use of amino acids, ability to use nitrate as an electron acceptor, and increased antibiotic resistance [87, 92]. While quorum sensing changes have been seen in *Burkholderia* spp. infections, inactivation may not be the norm in long-term infections [93].

S. aureus biofilms produce more heterogeneous biofilms in CF that involve both adhesive proteins, surface polysaccharides such as poly-*N*-acetyl-1,6 glucosamine surface polysaccharide (PIA/PNAG), wall teichoic acids, and DNA with the relative importance of each varying between strains [94]. Activation, of the quorum sensing system *agr* in *S. aureus* leads to biofilm dispersal and it is notable that *agr* mutants are often found in CF samples. In biofilms, *agr* transcriptional activity is low, and *agr* mutants are known to be enhanced for biofilm formation [95].

OTHER TYPES OF SMALL COLONY VARIANTS, AUXOTROPHS, AND METABOLISM

Confusingly, the small colony phenotype (SCV) that arises in *S. aureus* from CF patients appears to have a very different biological underpinning than the RSCVs noted above for *P. aeruginosa* [96–99]. *S. aureus* SCVs have several critical advantages as residents of the CF lung including higher tolerance to antibiotic challenges, and increased intracellular survival [94]. These strains often have slower growth, decreased hemolysin production, less pigmentation, increased intracellular survival, and inactive *agr* systems all suggesting a quiescent, biofilm forming lifestyle [94]. The genetic basis of SCVs is heterogeneous, and the associated mutations cause defects in amino acid or nucleotide synthesis and electron transport. SCVs are often auxotrophs, meaning that they cannot synthesize critical molecules needed for growth, which they need to acquire from exogenous sources. *S. aureus* SCVs are most usually auxotrophs of the nucleic acid thymidine with mutations in the *thyA* gene [100, 101]. These isolates are associated with long-term trimethoprim sulfamethoxazole treatment, are found mostly in older patients, tend to be have higher rates of antibiotic resistance, and have long-term persistence [98, 100, 102]. More recently, *S. aureus* SCVs have been found to be strongly associated with worse lung function [103, 104]. An interesting observation has been that interactions between *P. aeruginosa* and *S. aureus* may select for *S. aureus* SCVs and protecting them from toxicity of the very commonly used aminoglycoside tobramycin [105].

S. maltophilia has been reported to produce small colony variants that seem more similar to *S. aureus* SCVs than those reported for *P. aeruginosa* or *Burkholderia* spp. These are auxotrophs for hemin, methionine and thymidine and are associated with trimethoprim sulfamethoxazole exposure [106].

Auxotrophy also develops in *P. aeruginosa* isolates from CF, which mostly require amino acids (methionine, lysine, arginine) for growth [107–109]. It has been hypothesized that these forms can arise because the high levels of amino acids in the lung remove selection pressure [110]. Metabolomic studies have also shown evidence of metabolic adaptation with acetate production being negatively correlated with length of infection, and an increase in efficient uptake of amino acids that would be found in the human lung [111]

REACTIVE OXYGEN SPECIES

In addition to exogenous antibiotics, bacteria face an environment that is replete with damaging molecules released from neutrophils such as myeloperoxidase, elastase, leukocyte protease, and ROS [112, 113]. In general, bacteria must develop strategies to ameliorate this damage. Adaptive mutations for the oxidative stress (in *katG*, *yedY*, *moeA*) have been documented in *Burkholderia* cenocepacia [114]. One metabolic change noted in *P. aeruginosa* is mutations in the genes for the pyruvate dehydrogenase (*aceE* and *aceF*), which through reduction of glycolysis and the tricarboxylic acid (TCA) cycle can help resist reactive oxygen species [14, 115].

Because ROS can damage DNA, chronic ROS exposure can lead to mutations in many genes, which may happen to be in DNA repair genes that increase mutation rates even further [116]. Reactive oxygen stress has been shown to lead to mutations that create mucoid strains [117], and other experimental evolution studies done with chronic H2O2 have shown generation of mutants that result in a rough small colony variant phenotype (mediated by the *wspF* gene) [118]. However, some mucoid mutants have been shown to be even more susceptible to reactive oxygen stress [119].

HYPERMUTATION AND GENOME DEGRADATION

A recurrent theme in intra-host evolution is the generation of strains with higher rates of mutation. This phenomenon has been observed or inferred by sequence changes in *P. aeruginosa* [116, 120–125], *Haemophilus influenzae* [126], *Burkholderia* spp. ([12, 23, 127, 128] though see [129]) and *S. aureus* [26, 94] with the consequences being more rapid gain of mutations associated with antibiotic resistance [120] and other potentially beneficial traits. The genetic changes associated with hypermutability are associated with deletion or inactivation of DNA mismatch repair (MMR) genes (*mutLS, uvrD*) or the GO system (*mutMTY*), of oxidative damage repair and have been described in both gram-positive and negative CF pathogens [130–132].

Of course, while hypermutability offers a more rapid generation of beneficial point mutations, it is more likely to result in deleterious mutations. Therefore, the fitness cost of a

hypermutable phenotype is high [124] though this may be offset by other benefits in certain selective regimes [133]. It is interesting to speculate that a strategy that allows for a transient increase in mutation rate might be ideal. In this regard, it would be interesting to find evidence of restored MutLS activity, or antimutator phenotypes [134].

An area that has only started to be considered is the impact of recombination on rapid DNA change. If foreign DNA is available, then recombination is the fastest way to affect massive genomic change. One study of a single CF patient with chronic *B. multivorans* infection showed a significant genomic signal supporting recombination of more than 15% of the genomic sequence [23]. In *P. aeruginosa,* recombination as the dominant mode of sequence change has also been detected [135, 136].

Another mode of evolution is through insertion of transposable elements insertion elements (IS). Evolution through active IS elements in *P. aeruginosa* during CF infection has aids in the genesis of antibiotic resistance and the inactivation of genes [137–141]. In *B. cenocepacia*, transposition of IS elements leads both to gene inactivation if inserted into a coding or regulatory sequence, and also as loci of sequence homology that can undergo recombination leading to large genomic deletions [114, 129].

The combined action of mutation, transposition, and recombination in CF leads to genome degradation that is often seen as organisms become specialists, adapting to a specific environment where a more diverse set of genes (and possible functions) is no longer needed [141].

DIVERGENCE AND THE MAINTENANCE OF DIVERSITY

Divergence of the initial infecting population and long-term persistence of that diversity is another common pattern in CF [12, 142, 143], which signals that the CF lung environment furnishes positive (or diversifying) selective forces [12, 144] as well the potential that selective forces work differently on closely related bacteria. For *P. aeruginosa*, multiple studies have shown a large amount of phenotypic diversity in sputum samples [16–18, 142, 143, 145–147], which has also been shown in other pathogens including *Burkholderia* [148, 149], and at least in one case of nontuberculous mycobacteria [150]. Diversity in *S. aureus* has been less well characterized with only a handful of studies characterizing within-host or longitudinal diversity [151–154].

What evolutionary forces generate this diversity? One possibility is that distinct changes occur in distinct biogeographical locations in the lung where populations experience different conditions as well as the stochastic effects associated with population bottlenecks [16, 155]. While anatomical regions can create subdivisions in bacterial populations, it is also possible that there are smaller subdivisions such as aggregates of bacteria or even in different regions of the airway lumen [141, 156]. In a subdivided population the existence of periodic selection that occurs with intermittent use of antibiotics, or recurrent periods of inflammation and immune response, might lead to selective sweeps, in which variants arise from mutation that dominate the population because of their increased fitness [157]. A subdivided population also favors diversification through genetic drift over selection [141].

An alternative explanation for increased and stable diversity is that the organisms with different phenotypes could be occupying different niches within the lung, however testing this hypothesis will require demonstrating differences in spatial organization or nutrient utilization.

Yet another possibility is that diversity arises from social interactions between organisms, which could take the form of mutualisms (divisions of labor) or unequal relationships such as with social cheaters who acquire benefits from the population while reducing any cost to themselves [158]. Interesting new work on *P. aeruginosa* populations suggests that genes for relatively cooperative functions are more likely to vary and have inactivating mutations than genes that largely function in the context of one bacterium, suggesting that social interactions between bacteria may be critical for diversification [159].

HOW DOES ADAPTATION AFFECT HEALTH AND TREATMENT?

Many studies use the word "pathoadaptation" to describe the process of adaptation to the host environment, but this term can be confusing since it also implies enhanced fitness that leads to worse disease [160, 161]. In the sense that the increased fitness of specific phenotypic changes make infections last longer than probably all in host adaptation can be considered pathoadaptation [162]. However, some phenotypic changes may decrease or change the overall virulence of the organism [163]. For instance, some hypermutator phenotypes have been directly tied to increased persistence but reduced or changed virulence [164].

The phenotypic differences that are well documented for *P. aeruginosa* can lead to significant differences in antibiotic susceptibility, which has an impact on susceptibility testing and likely treatment as well [165–168]. Similar diversity has been noted in nontuberculous mycobacteria isolated from the same individual [150] as well as in *S. maltophilia* [169], and it is probably a general feature of chronic lung infections.

A recent report [170] highlights the possibility that mutations can result in increased pathogenicity and antibiotic resistance. In this case report, whole genome sequencing revealed mutations in the *exsD* gene whose product acts to negatively regulate type III secretion (T3S), a major virulence factor for *P. aeruginosa* that is often lost earlier in infection and may be linked to worse outcomes [171, 172]. The authors note that this mutation could have led to hyperactivity of T3S that coincided with an acute

worsening in the patient's status. Interestingly, mutation of the OprD porin was also observed in this report, which likely had an impact on the susceptibility of this strain to carbapenem antibiotics, suggesting that the *exsD* mutation may have genetically "hitch–hiked" along with an antibiotic resistance gene.

This example evokes themes that repeatedly confound our ability to gauge the impact of mutations and putative adaptation in cystic fibrosis. First, adaptations tend to occur later in disease when lung function is already declining, thus it is not always clear whether adaptation is causal or just a marker worsening disease. Second, mutations occur linked to one another in a genomic (and even microbial population) context. Therefore, it may be difficult to discern which mutations are beneficial to the organism, which are harmful to the host, and which are neutral or even beneficial to the host. As such, while phenotypes such as the mucoid phenotype in *P. aeruginosa* [6, 162, 173–183] or the SCV phenotype [103, 104] in *S. aureus* are strongly associated with worsening disease, it is difficult to assign them a causal relationship. Likewise, associations between *P. aeruginosa* auxotrophs, RSCVs, and quorum sensing and more severe disease remain associations [79, 102, 108, 184, 185].

It is also interesting to consider that some of the mutations that occur may actually lead to less inflammatory or destructive phenotypes. For instance, the loss of flagella might lead to less inflammation due to the immune response through TLR5 [37] or the differences in Lipid A acylation might decrease inflammation due to TLR4 signaling [42–46]. There have been no clinical studies to gauge the impact of these changes on disease progression.

WHAT IS NEXT?

As genomic and phenotypic data accumulate the next steps will be to clearly delineate the impact of bacterial adaptations on disease with the goal of using this information to make clinical decisions and interventions. The critical clinical studies will include careful determination of which adaptations are associated with clinical decline, and which are not. This will require large-scale studies that consider intra-host diversity and change over time. Once these associations are well established, they can be targeted with treatments and decision making. For instance, it may be possible to target specific phenotypes to aid in eradication of adapted strains. It may also be possible to monitor for specific adaptations to guide clinical decision making. Some adaptations may signal the need for more aggressive treatment, and some may change the risk benefit analysis of aggressive therapy leading to more judicious use of antibiotics or other drugs. These types of approaches will require truly translational research that includes rigorous genomic, basic, and clinical studies. There should also be more attention paid to emerging and less well-studied pathogens such as nontuberculous mycobacteria, *S. maltophilia*, and *S. aureus*, as well as other underappreciated members of the CF

respiratory microbiome, such as anaerobes. It is important to note that microbial adaptations not only allow us to understand the process of intra-host evolution, but they also give a window into the underlying biology of the disease.

References

1. Doggett RG, Harrison GM, and Wallis ES. Comparison of some properties of *Pseudomonas aeruginosa* isolated from infections in persons with and without cystic fibrosis. *J Bacteriol* 1964; 87:427–31.
2. Govan JR and Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* 1996. 60(3):539–74.
3. Evans LR and Linker A. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. *J Bacteriol* 1973. 116(2):915–24.
4. Linker A and Jones RS. A new polysaccharide resembling alginic acid isolated from Pseudomonads. *J Biol Chem* 1966. 241: 3845–3851.
5. Stanford ECC. On algin: a new substance obtained from some of the commoner species of marine algae. *Chemical News* 1883(47):254–257.
6. Pedersen SS. Lung infection with alginate–producing, mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *APMIS Suppl* 1992. 28:1–79.
7. Ryder C, Byrd M and Wozniak DJ. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr Opin Microbiol* 2007. 10(6):644–8.
8. Lieberman TD, et al., Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. *Nat Genet* 2011. 43(12):1275–80.
9. Smith EE, et al., Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A* 2006. 103(22):8487–92.
10. Marvig RL, et al., Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat Genet* 2015. 47(1):57–64.
11. Folkesson A, et al., Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. *Nat Rev Microbiol* 2012. 10(12):841–51.
12. Silva IN, et al., Long-term evolution of *Burkholderia multivorans* during a chronic cystic fibrosis infection reveals shifting forces of selection. *mSystems* 2016. 1(3):00029–16.
13. Huse HK, et al., Parallel evolution in *Pseudomonas aeruginosa* over 39,000 generations in vivo. *mBio*, 2010. 1(4):00199–10.
14. Rossi E, et al., *Pseudomonas aeruginosa* adaptation and evolution in patients with cystic fibrosis. *Nat Rev Microbiol* 2021. 19(5):331–342.
15. Lagrange–Puget M, et al., Longitudinal study of oxidative status in 312 cystic fibrosis patients in stable state and during bronchial exacerbation. *Pediatr Pulmonol* 2004. 38(1):43–9.
16. Jorth P, et al., *Regional* isolation drives bacterial diversification within cystic fibrosis lungs. *Cell Host Microbe* 2015. 18(3):307–19.
17. Kordes A, et al., Genetically diverse *Pseudomonas aeruginosa* populations display similar transcriptomic profiles in a cystic fibrosis explanted lung. *Nat Commun* 2019. 10(1):3397.
18. Willner D, et al., Spatial distribution of microbial communities in the cystic fibrosis lung. *ISME J* 2012. 6(2):471–4.
19. Pitt TL, et al., Survey of resistance of *Pseudomonas aeruginosa* from UK patients with cystic fibrosis to six commonly prescribed antimicrobial agents. *Thorax* 2003; 58(9):794–6.
20. Cheng K, et al., Spread of beta–lactam–resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet* 1996; 348(9028):639–42.
21. Lechtzin N, et al., Outcomes of adults with cystic fibrosis infected with antibiotic–resistant *Pseudomonas aeruginosa*. *Respiration* 2006; 73(1):27–33.
22. Langendonk RF, Neill DR and Fothergill JL. The building blocks of antimicrobial resistance in *Pseudomonas aeruginosa*: implications for current resistance–breaking therapies. *Front Cell Infect Microbiol*, 2021; 11:665759.

23. Diaz Caballero J, et al., A genome–wide association analysis reveals a potential role for recombination in the evolution of antimicrobial resistance in *Burkholderia multivorans*. *PLoS Pathog*, **2018**; 14(12):e1007453e1007453.

24. Scoffone VC, et al., Role of RND efflux pumps in drug resistance of cystic fibrosis pathogens. *Antibiotics (Basel)*, **2021**; 10(7).

25. Kim SY, et al., Nontuberculous mycobacterial lung disease: ecology, microbiology, pathogenesis, and antibiotic resistance mechanisms. *Precision Future Med*, **2017**; 1(3):99–114.

26. Prunier AL, et al., High rate of macrolide resistance in *Staphylococcus aureus* strains from patients with cystic fibrosis reveals high proportions of hypermutable strains. *J Infect Dis*, **2003**; 187(11):1709–16.

27. Mah TF and O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **2001**; 9(1):34–9.

28. Xu KD, McFeters GA, and Stewart PS. Biofilm resistance to antimicrobial agents. *Microbiology (Reading)* **2000**; 146 (Pt 3):547–549.

29. Hoiby N, Ciofu O and Bjarnsholt T. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol*, **2010**; 5(11):1663–74.

30. Head NE and Yu H. Cross-sectional analysis of clinical and environmental isolates of *Pseudomonas aeruginosa*: biofilm formation, virulence, and genome diversity. *Infect Immun* **2004**; 72(1):133–44.

31. Malone JG. Role of small colony variants in persistence of *Pseudomonas aeruginosa* infections in cystic fibrosis lungs. *Infect Drug Resist*, **2015**; 8:237–47.

32. Bartell JA, et al., Bacterial persisters in long–term infection: emergence and fitness in a complex host environment. *PLoS Pathog*, **2020**; 16(12):e1009112e1009112.

33. La Rosa R, et al., Compensatory evolution of *Pseudomonas aeruginosa's* slow growth phenotype suggests mechanisms of adaptation in cystic fibrosis. *Nat Commun* **2021**; 12(1):3186.

34. Pandey S, Kawai T and Akira S. Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors. *Cold Spring Harb Perspect Biol* **2014**; 7(1):a016246a016246.

35. Feldman M, et al., Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* **1998**; 66(1):43–51.

36. Mahenthiralingam E, Campbell ME, and Speert DP. Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun* **1994**; 62(2):596–605.

37. Zhang J, et al., Toll-like receptor 5-mediated corneal epithelial inflammatory responses to *Pseudomonas aeruginosa* flagellin. *Invest Ophthalmol Vis Sci* **2003**; 44(10):4247–54.

38. Lee B, et al., Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *J Clin Microbiol*, **2005**; 43(10): 5247–55.

39. Kresse AU, et al., Impact of large chromosomal inversions on the adaptation and evolution of *Pseudomonas aeruginosa* chronically colonizing cystic fibrosis lungs. *Mol Microbiol* **2003**; 47(1):145–58.

40. Hales BA, et al., Variation in flagellin genes and proteins of *Burkholderia cepacia*. *J Bacteriol* **1998**; 180(5):1110–8.

41. Zlosnik JE, et al., Swimming motility in a longitudinal collection of clinical isolates of *Burkholderia cepacia* complex bacteria from people with cystic fibrosis. *PLoS One* **2014**; 9(9):e106428.

42. Cigana C, et al., *Pseudomonas aeruginosa* exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. *PLoS One* **2009**; 4(12):e8439e8439.

43. Ernst RK, et al., Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* **1999**; 286(5444):1561–5.

44. Ernst RK, et al., Unique lipid a modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. *J Infect Dis* **2007**; 196(7):1088–92.

45. Ernst RK, et al., *Pseudomonas aeruginosa* lipid A diversity and its recognition by Toll-like receptor 4. *J Endotoxin Res* **2003**; 9(6):395–400.

46. Ernst RK, et al., The *Pseudomonas aeruginosa* lipid A deacylase: selection for expression and loss within the cystic fibrosis airway. *J Bacteriol* **2006**; 188(1):191–201.

47. Lam MY, et al., Occurrence of a common lipopolysaccharide antigen in standard and clinical strains of *Pseudomonas aeruginosa*. *J Clin Microbiol* **1989**; 27(5):962–7.

48. Hancock RE, et al., *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum–sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect Immun* **1983**; 42(1):170–7.

49. Hassan AA, Coutinho CP, and Sa-Correia I. *Burkholderia cepacia* complex species differ in the frequency of variation of the lipopolysaccharide O–antigen expression during cystic fibrosis chronic respiratory infection. *Front Cell Infect Microbiol* **2019**; 9:273.

50. Reyes RE, et al., Mechanisms of O-antigen structural variation of bacterial lipopolysaccharide (LPS). In: Karunaratne DN, editor. The Complex World of Polysaccharides [Internet]. London: IntechOpen; **2012** [cited 2022 Jul 22]. Available at: https://www.intechopen.com/chapters/40579.

51. Schmerk, CL and Valvano MA. Burkholderia multivorans survival and trafficking within macrophages. *J Med Microbiol* **2013**; 62(Pt 2):173–184.

52. Gutierrez AV, et al., Glycopeptidolipids, a double–edged sword of the *Mycobacterium abscessus* complex. *Front Microbiol* **2018**; 9:1145.

53. Scribner MR, et al., The nutritional environment is sufficient to select coexisting biofilm and quorum sensing mutants of *Pseudomonas aeruginosa*. *J Bacteriol* **2022**; 204(3):e0044421.

54. Traverse CC, et al., Tangled bank of experimentally evolved *Burkholderia* biofilms reflects selection during chronic infections. *Proc Natl Acad Sci U S A* **2013**; 110(3):E250–9.

55. Schurr MJ, et al., The algD promoter: regulation of alginate production by *Pseudomonas aeruginosa* in cystic fibrosis. *Cell Mol Biol Res* **1993**; 39(4):371–6.

56. Schurr MJ, et al., Gene cluster controlling conversion to alginate–overproducing phenotype in *Pseudomonas aeruginosa:* functional analysis in a heterologous host and role in the instability of mucoidy. *J Bacteriol*, **1994**; 176(11):3375–82.

57. Hoyle BD and Costerton JW. Bacterial resistance to antibiotics: the role of biofilms. *Prog Drug Res*, **1991**; 37:91–105.

58. Stewart PS and Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet*, **2001**; 358(9276):135–8.

59. Conway BA, Venu V and Speert DP. Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. *J Bacteriol*, **2002**; 184(20):5678–85.

60. Huber B, et al., Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. *Mol Microbiol*, **2002**; 46(2):411–26.

61. Huber B, et al., The cep quorum–sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology (Reading)*, **2001**; 147(Pt 9):2517–2528.

62. Richau JA, et al., Molecular typing and exopolysaccharide biosynthesis of *Burkholderia cepacia* isolates from a Portuguese cystic fibrosis center. *J Clin Microbiol*, **2000**; 38(4):1651–5.

63. Cunha MV, et al., Studies on the involvement of the exopolysaccharide produced by cystic fibrosis–associated isolates of the *Burkholderia cepacia* complex in biofilm formation and in persistence of respiratory infections. *J Clin Microbiol*, **2004**; 42(7):3052–8.

64. Zlosnik JE, et al., Differential mucoid exopolysaccharide production by members of the *Burkholderia cepacia* complex. *J Clin Microbiol*, **2008**; 46(4):1470–3.

65. Cerantola S and Montrozier H. Production in vitro, on different solid culture media, of two distinct exopolysaccharides by a mucoid clinical strain of *Burkholderia cepacia*. *FEMS Microbiol Lett*, **2001**; 202(1):129–33.

66. Cerantola S, et al., Exopolysaccharide production by mucoid and non–mucoid strains of *Burkholderia cepacia*. *FEMS Microbiol Lett*, **2000**; 185(2):243–6.

67. Sist P, et al., Macromolecular and solution properties of Cepacian: the exopolysaccharide produced by a strain of *Burkholderia cepacia* isolated from a cystic fibrosis patient. *Carbohydr Res*, **2003**; 338(18):1861–7.

68. Moreira LM, et al., Identification and physical organization of the gene cluster involved in the biosynthesis of *Burkholderia cepacia* complex exopolysaccharide. *Biochem Biophys Res Commun*, **2003**; 312(2):323–33.

69. Chung JW, et al., Colonial morphology of *Burkholderia cepacia* complex genomovar III: implications in exopolysaccharide production, pilus expression, and persistence in the mouse. *Infect Immun*, **2003**; 71(2):904–9.

70. Bylund J, et al., Exopolysaccharides from *Burkholderia cenocepacia* inhibit neutrophil chemotaxis and scavenge reactive oxygen species. *J Biol Chem*, **2006**; 281(5):2526–32.

71. Zlosnik JE, et al., Mucoid and nonmucoid *Burkholderia cepacia* complex bacteria in cystic fibrosis infections. *Am J Respir Crit Care Med*, **2011**; 183(1):67–72.

72. Kovach K, et al., Evolutionary adaptations of biofilms infecting cystic fibrosis lungs promote mechanical toughness by adjusting polysaccharide production. *NPJ Biofilms Microbiomes*, **2017**; 3:1.

73. Wozniak DJ, et al., Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A*, **2003**; 100(3):7907–12.

74. Periasamy S, et al., How *Staphylococcus aureus* biofilms develop their characteristic structure. *Proc Natl Acad Sci U S A*, **2012**; 109(4):1281–6.

75. Harrison JJ, et al., Elevated exopolysaccharide levels in *Pseudomonas aeruginosa* flagellar mutants have implications for biofilm growth and chronic infections. *PLoS Genet*, **2020**; 16(6):e1008848.

76. Starkey M, et al., *Pseudomonas aeruginosa* rugose small–colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *J Bacteriol*, **2009**; 191(11):3492–503.

77. Huse HK, et al., *Pseudomonas aeruginosa* enhances production of a non–alginate exopolysaccharide during long–term colonization of the cystic fibrosis lung. *PLoS One*, **2013**; 8(12):e82621.

78. Haussler S, et al., Highly adherent small–colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J Med Microbiol*, **2003**; 52(Pt 4):295–301.

79. Haussler S, et al., Small–colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. *Clin Infect Dis*, **1999**; 29(3):621–5.

80. Malone JG, et al., YfiBNR mediates cyclic di–GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. *PLoS Pathog*, **2010**; 6(3):e1000804.

81. Malone JG, et al., The YfiBNR signal transduction mechanism reveals novel targets for the evolution of persistent *Pseudomonas aeruginosa* in cystic fibrosis airways. *PLoS Pathog*, **2012**; 8(6):e1002760.

82. Drenkard E and Ausubel FM. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature*, **2002**; 416(6882):740–3.

83. D'Argenio DA, et al., Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. *J Bacteriol*, **2002**; 184(23):6481–9.

84. Cooper VS, et al., Parallel evolution of small colony variants in *Burkholderia cenocepacia* biofilms. *Genomics*, **2014**; 104(6 Pt A):447–52.

85. Haussler S, et al., Fatal outcome of lung transplantation in cystic fibrosis patients due to small–colony variants of the *Burkholderia cepacia* complex. *Eur J Clin Microbiol Infect Dis*, **2003**; 22(4):249–53.

86. Parsek MR and Greenberg EP. Acyl–homoserine lactone quorum sensing in gram–negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc Natl Acad Sci U S A*, **2000**; 97(16):8789–93.

87. D'Argenio DA, et al., Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol*, **2007**; 64(2):512–33.

88. Wilder CN, Allada G and Schuster M. Instantaneous within–patient diversity of *Pseudomonas aeruginosa* quorum–sensing populations from cystic fibrosis lung infections. *Infect Immun*, **2009**; 77(12):5631–9.

89. Bjarnsholt T, et al., Quorum sensing and virulence of *Pseudomonas aeruginosa* during lung infection of cystic fibrosis patients. *PLoS One*, **2010**; 5(4):e10115.

90. Girard G and Bloemberg GV. Central role of quorum sensing in regulating the production of pathogenicity factors in *Pseudomonas aeruginosa*. *Future Microbiol*, **2008**; 3(1):97–106.

91. Schaber JA, et al., Analysis of quorum sensing–deficient clinical isolates of *Pseudomonas aeruginosa*. *J Med Microbiol*, **2004**; 53(Pt 9):841–853.

92. Hoffman LR, Nutrient availability as a mechanism for selection of antibiotic tolerant *Pseudomonas aeruginosa* within the CF airway. *PLoS Pathog*, **2010**; 6(1):e1000712.

93. McKeon SA, et al., Functional quorum sensing systems are maintained during chronic *Burkholderia cepacia* complex infections in patients with cystic fibrosis. *J Infect Dis*, **2011**; 203(3):383–92.

94. Goerke C and Wolz C. Adaptation of *Staphylococcus aureus* to the cystic fibrosis lung. *Int J Med Microbiol*, **2010**; 300(8):520–5.

95. Goerke C, et al., Direct quantitative transcript analysis of the agr regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro. *Infect Immun*, **2000**; 68(3):1304–11.

96. Proctor RA and Peters G. Small colony variants in staphylococcal infections: diagnostic and therapeutic implications. *Clin Infect Dis*, **1998**; 27(3):419–22.

97. Proctor RA, et al., Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol*, **2006**; 4(4):295–305.

98. Kahl B, et al., Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *J Infect Dis*, **1998**; 177(4):1023–9.

99. Moisan H, et al., Transcription of virulence factors in *Staphylococcus aureus* small–colony variants isolated from cystic fibrosis patients is influenced by SigB. *J Bacteriol*, **2006**; 188(1):64–76.

100. Besier, S, et al., Molecular analysis of the thymidine–auxotrophic small colony variant phenotype of *Staphylococcus aureus*. *Int J Med Microbiol*, **2007**; 297(4):217–25.

101. Chatterjee I, et al., In vivo mutations of thymidylate synthase (encoded by thyA) are responsible for thymidine dependency in clinical small–colony variants of *Staphylococcus aureus*. *J Bacteriol*, **2008**; 190(3):834–42.

102. Schneider M, et al., Clinical characteristics associated with isolation of small–colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. *J Clin Microbiol*, **2008**; 46(5):1832–4.

103. Wolter DJ, et al., *Staphylococcus aureus* small–colony variants are independently associated with worse lung disease in children with cystic fibrosis. *Clin Infect Dis*, **2013**; 57(3):384–91.

104. Wolter DJ, et al., Prevalence and clinical associations of *Staphylococcus aureus* small–colony variant respiratory infection in children with cystic fibrosis (SCVSA): a multicentre, observational study. *Lancet Respir Med* **2019**; 7(12):1027–1038.

105. Hoffman LR, et al., Selection for *Staphylococcus aureus* small–colony variants due to growth in the presence of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **2006**; 103(52):19890–5.

106. Anderson SW, et al., Characterization of small–colony–variant *Stenotrophomonas maltophilia* isolated from the sputum specimens of five patients with cystic fibrosis. *J Clin Microbiol* **2007**; 45(2):529–35.

107. Barth AL and Pitt TL. Auxotrophic variants of *Pseudomonas aeruginosa* are selected from prototrophic wild–type strains in respiratory infections in patients with cystic fibrosis. *J Clin Microbiol* **1995**; 33(1):37–40.

108. Taylor RF, Hodson ME and Pitt TL. Adult cystic fibrosis: association of acute pulmonary exacerbations and increasing severity of lung disease with auxotrophic mutants of *Pseudomonas aeruginosa*. *Thorax* **1993**; 48(10):1002–5.

109. Taylor RF, Hodson ME and Pitt TL. Auxotrophy of *Pseudomonas aeruginosa* in cystic fibrosis. *FEMS Microbiol Lett*, **1992**; 71(3):243–6.

110. Barth AL and Pitt TL. The high amino–acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic *Pseudomonas aeruginosa*. *J Med Microbiol*, **1996**; 45(2):110–9.

111. Behrends V, et al., Metabolic adaptations of *Pseudomonas aeruginosa* during cystic fibrosis chronic lung infections. *Environ Microbiol*, **2013**; 15(2):398–408.

112. Hull J, et al., Pulmonary oxidative stress response in young children with cystic fibrosis. *Thorax*, **1997**; 52(6):557–60.

113. Doring G, et al., Elastase from polymorphonuclear leucocytes: a regulatory enzyme in immune complex disease. *Clin Exp Immunol*, **1986**; 64(3):597–605.

114. Nunvar J, et al., What matters in chronic *Burkholderia cenocepacia* infection in cystic fibrosis: Insights from comparative genomics. *PLoS Pathog*, **2017**; 13(12):e1006762.

115. Rossi E, et al., High–resolution in situ transcriptomics of *Pseudomonas aeruginosa* unveils genotype independent patho–phenotypes in cystic fibrosis lungs. *Nat Commun*, **2018**; 9(1):3459.

116. Ciofu O, et al., Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother*, **2005**; 49(6):2276–82.

117. Mathee K, et al., Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology (Reading)*, **1999**; 145 (Pt 6):1349–1357.

118. Chua SL, et al., Reactive oxygen species drive evolution of pro–biofilm variants in pathogens by modulating cyclic–di–GMP levels. *Open Biol*, **2016**; 6(11):160162.

119. Boucher JC, et al., Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. *J Bacteriol*, **1996**; 178(2):511–23.

120. Oliver A, et al., Hypermutation and the preexistence of antibiotic–resistant *Pseudomonas aeruginosa* mutants: implications for susceptibility testing and treatment of chronic infections. *Antimicrob Agents Chemother*, **2004**; 48(11):4226–33.

121. Oliver A, et al., High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science*, **2000**; 288(5469):1251–4.

122. Colque CA, et al., Hypermutator *Pseudomonas aeruginosa* exploits multiple genetic pathways to develop multidrug resistance during long–term infections in the airways of cystic fibrosis patients. *Antimicrob Agents Chemother*, **2020**; 64(5):e02142-19.

123. Marvig RL, et al., Genome analysis of a transmissible lineage of *Pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS Genet*, **2013**; 9(9):e1003741.

124. Montanari S, et al., Biological cost of hypermutation in *Pseudomonas aeruginosa* strains from patients with cystic fibrosis. *Microbiology (Reading)*, **2007**; 153(Pt 5):1445–1454.

125. Hogardt M, et al., Stage–specific adaptation of hypermutable *Pseudomonas aeruginosa* isolates during chronic pulmonary infection in patients with cystic fibrosis. *J Infect Dis*, **2007**; 195(1):70–80.

126. Roman F, et al., Dynamics of long–term colonization of respiratory tract by *Haemophilus influenzae* in cystic fibrosis patients shows a marked increase in hypermutable strains. *J Clin Microbiol*, **2004**; 42(4):1450–9.

127. Viberg LT, et al., Within–Host Evolution of *Burkholderia pseudomallei* during chronic infection of seven Australasian cystic fibrosis patients. *mBio*, **2017**. 8(2):00356–17.

128. Martina P, et al., Hypermutation in *Burkholderia cepacia* complex is mediated by DNA mismatch repair inactivation and is highly prevalent in cystic fibrosis chronic respiratory infection. *Int J Med Microbiol*, **2014**; 304(8):1182–91.

129. Lood C, et al., Genomics of an endemic cystic fibrosis *Burkholderia multivorans* strain reveals low within–patient evolution but high between–patient diversity. *PLoS Pathog*, **2021**; 17(3):e1009418.

130. Oliver A, Sanchez JM and Blazquez J. Characterization of the GO system of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett*, **2002**; 217(1):31–5.

131. Oliver A, Baquero F and Blazquez J. The mismatch repair system (mutS, mutL and uvrD genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol Microbiol*, **2002**; 43(6):1641–50.

132. Miller JH. Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. *Annu Rev Microbiol*, **1996**; 50:625–43.

133. Alcala–Franco B, et al., Antibiotic pressure compensates the biological cost associated with *Pseudomonas aeruginosa* hypermutable phenotypes in vitro and in a murine model of chronic airways infection. *J Antimicrob Chemother*, **2012**; 67(4):962–9.

134. Wielgoss S, et al., Mutation rate dynamics in a bacterial population reflect tension between adaptation and genetic load. *Proc Natl Acad Sci U S A*, **2013**; 110(1):222–7.

135. Darch SE and Whiteley M. Show me the SNPs. How bacterial sex generates diversity in the cystic fibrosis lung. *Am J Respir Crit Care Med*, **2015**; 191(7):725–7.

136. Darch SE, et al., Recombination is a key driver of genomic and phenotypic diversity in a *Pseudomonas aeruginosa* population during cystic fibrosis infection. *Sci Rep*, **2015**; 5:7649.

137. Kresse AU, Blocker H and Romling U. ISPa20 advances the individual evolution of *Pseudomonas aeruginosa* clone C subclone C13 strains isolated from cystic fibrosis patients by insertional mutagenesis and genomic rearrangements. *Arch Microbiol*, **2006**; 185(4):245–54.

138. Wolter DJ, Hanson ND and Lister PD. Insertional inactivation of *oprD* in clinical isolates of *Pseudomonas aeruginosa* leading to carbapenem resistance. *FEMS Microbiol Lett*, **2004**; 236(1):137–43.

139. Evans JC and Segal H. A novel insertion sequence, ISPA26, in oprD of *Pseudomonas aeruginosa* is associated with carbapenem resistance. *Antimicrob Agents Chemother*, **2007**; 51(10):3776–7.

140. Sentausa E, et al., Insertion sequences drive the emergence of a highly adapted human pathogen. *Microb Genom*, **2020**; 6(9):mgen000265.

141. Armbruster CR, et al., Adaptation and genomic erosion in fragmented *Pseudomonas aeruginosa* populations in the sinuses of people with cystic fibrosis. *Cell Rep*, **2021**; 37(3):109829.

142. Clark ST, et al., Phenotypic diversity within a *Pseudomonas aeruginosa* population infecting an adult with cystic fibrosis. *Sci Rep*, **2015**; 5:10932.

143. Mowat E, et al., *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. *Am J Respir Crit Care Med* **2011**; 183(12):1674–9.

144. Dettman JR and Kassen R. Evolutionary genomics of niche–specific adaptation to the cystic fibrosis lung in *Pseudomonas aeruginosa*. *Mol Biol Evol*, **2021**; 38(2):663–675.

145. Workentine ML, et al., Phenotypic heterogeneity of *Pseudomonas aeruginosa* populations in a cystic fibrosis patient. *PLoS One*, **2013**; 8(4):e60225.

146. Workentine ML, et al., Phenotypic and metabolic profiling of colony morphology variants evolved from *Pseudomonas fluorescens* biofilms. *Environ Microbiol*, **2010**; 12(6):1565–77.

147. Ashish A, et al., Extensive diversification is a common feature of *Pseudomonas aeruginosa* populations during respiratory infections in cystic fibrosis. *J Cyst Fibros* **2013**; 12(6):790–3.

148. Lieberman TD, et al., Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures. *Nat Genet*, **2014**; 46(1):82–7.

149. Lee AH, et al., Phenotypic diversity and genotypic flexibility of *Burkholderia cenocepacia* during long–term chronic infection of cystic fibrosis lungs. *Genome Res*, **2017**; 27(4):650–662.

150. Shaw LP, et al., Children with cystic fibrosis are infected with multiple subpopulations of *Mycobacterium abscessus* with different antimicrobial resistance profiles. *Clin Infect Dis*, **2019**; 69(10):1678–1686.

151. Goerke C, et al., High phenotypic diversity in infecting but not in colonizing *Staphylococcus aureus* populations. *Environ Microbiol*, **2007**; 9(12):3134–42.

152. Bernardy EE, et al., Genotypic and phenotypic diversity of *Staphylococcus aureus* isolates from cystic fibrosis patient lung infections and their interactions with *Pseudomonas aeruginosa*. *mBio*, **2020**; 11(3):e00735–20.

153. Bernardy EE, et al., Whole–genome sequences of *Staphylococcus aureus* isolates from cystic fibrosis lung infections. *Microbiol Resour Announc*, **2019**; 8(3):e01564–18.

154. Azarian T, et al., Long–term intrahost evolution of methicillin resistant *Staphylococcus aureus* among cystic fibrosis patients with respiratory carriage. *Front Genet*, **2019**; 10:546.

155. Winstanley C, O'Brien S and Brockhurst MA. *Pseudomonas aeruginosa* evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. *Trends Microbiol*, **2016**; 24(5):327–337.

156. Bjarnsholt T, et al., The in vivo biofilm. *Trends Microbiol* **2013**; 21(9):466–474.

157. Diaz Caballero J, et al., Selective sweeps and parallel pathoadaptation drive *Pseudomonas aeruginosa* evolution in the cystic fibrosis lung. *mBio*, **2015**; 6(5):e00981–15.

158. West SA, et al., Social evolution theory for microorganisms. *Nat Rev Microbiol*, **2006**; 4(8):597–607.

159. Belcher LJ, et al., Kin selection for cooperation in natural bacterial populations. *Proc Natl Acad Sci U S A*, **2022**; 119(9).

160. Pallen MJ and Wren BW, Bacterial pathogenomics. *Nature*, **2007**; 449(7164):835–42.

161. Sokurenko EV, Hasty DL and Dykhuizen DE, Pathoadaptive mutations: gene loss and variation in bacterial pathogens. *Trends Microbiol*, **1999**; 7(5):191–5.

162. Hauser AR, et al., Clinical significance of microbial infection and adaptation in cystic fibrosis. *Clin Microbiol Rev*, **2011**; 24(1):29–70.

163. Bragonzi A, et al., *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. *Am J Respir Crit Care Med*, **2009**; 180(2):138–45.

164. Mena A, et al., Inactivation of the mismatch repair system in *Pseudomonas aeruginosa* attenuates virulence but favors persistence of oropharyngeal colonization in cystic fibrosis mice. *J Bacteriol*, **2007**; 189(9):3665–8.

165. Smith SR, et al., Impact of antibiotic–resistant pseudomonas on the survival of cystic fibrosis (CF) patients following heart–lung transplantation. *J Heart Lung Transplant* **2001**; 20(2):224.

166. Foweraker JE, et al., Comparison of methods to test antibiotic combinations against heterogeneous populations of multiresistant *Pseudomonas aeruginosa* from patients with acute infective exacerbations in cystic fibrosis. *Antimicrob Agents Chemother*, **2009**; 53(11):4809–15.

167. Foweraker JE, et al., Phenotypic variability of *Pseudomonas aeruginosa* in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. *J Antimicrob Chemother*, **2005**; 55(6):921–7.

168. Foweraker JE and Govan JR. Antibiotic susceptibility testing in early and chronic respiratory infections with *Pseudomonas aeruginosa*. *J Cyst Fibros*, **2013**; 12(3):302.

169. Esposito A, et al., Evolution of *Stenotrophomonas maltophilia* in cystic fibrosis lung over chronic infection: a genomic and phenotypic population study. *Front Microbiol*, **2017**; 8:1590.

170. Jorth P, et al., Cystic fibrosis lung function decline after within–host evolution increases virulence of infecting *Pseudomonas aeruginosa*. *Am J Respir Crit Care Med*, **2021**; 203(5):637–640.

171. Jain M, et al., Type III secretion phenotypes of *Pseudomonas aeruginosa* strains change during infection of individuals with cystic fibrosis. *J Clin Microbiol*, **2004**; 42(11):5229–37.

172. Jain M, et al., Evolution of *Pseudomonas aeruginosa* type III secretion in cystic fibrosis: a paradigm of chronic infection. *Transl Res*, **2008**; 152(6):257–64.

173. Johansen HK and Hoiby N. Seasonal onset of initial colonisation and chronic infection with *Pseudomonas aeruginosa* in patients with cystic fibrosis in Denmark. *Thorax*, **1992**; 47(2):109–11.

174. Pressler T, et al., Early rise of anti–pseudomonas antibodies and a mucoid phenotype of *Pseudomonas aeruginosa* are risk factors for development of chronic lung infection––a case control study. *J Cyst Fibros*, **2006**; 5(1):9–15.

175. Ballmann M, Rabsch P and von der Hardt H. Long–term follow up of changes in FEV1 and treatment intensity during *Pseudomonas aeruginosa* colonisation in patients with cystic fibrosis. *Thorax*, **1998**; 53(9):732–7.

176. Farrell PM, et al., Association between mucoid *Pseudomonas infection* and bronchiectasis in children with cystic fibrosis. *Radiology*, **2009**; 252(2):534–43.

177. Henry RL, Mellis CM and Petrovic L. Mucoid *Pseudomonas aeruginosa* is a marker of poor survival in cystic fibrosis. *Pediatr Pulmonol*, **1992**; 12(3):158–61.

178. Henry RL, et al., Mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Aust Paediatr J*, **1982**; 18(1):43–5.

179. Hoiby N, et al., *Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. *Scand J Respir Dis*, **1977**; 58(2):65–79.

180. Pedersen SS, et al., Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax*, **1992**; 47(1):6–13.

181. Demko CA, Byard PJ and Davis PB. Gender differences in cystic fibrosis: *Pseudomonas aeruginosa* infection. *J Clin Epidemiol* **1995**; 48(8):1041–9.

182. Li Z, et al., Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. *JAMA*, **2005**; 293(5):581–8.

183. Parad RB, et al., Pulmonary outcome in cystic fibrosis is influenced primarily by mucoid *Pseudomonas aeruginosa* infection and immune status and only modestly by genotype. *Infect Immun*, **1999**; 67(9):4744–50.

184. Thomas SR, et al., Increased sputum amino acid concentrations and auxotrophy of *Pseudomonas aeruginosa* in severe cystic fibrosis lung disease. *Thorax*, **2000**; 55(9):795–7.

185. Hoffman LR, et al., *Pseudomonas aeruginosa* lasR mutants are associated with cystic fibrosis lung disease progression. *J Cyst Fibros*, **2009**; 8(1):66–70.