Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective

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Abstract | The airways of patients with cystic fibrosis (CF) are nearly always infected with many different microorganisms. This environment offers warm, humid and nutrient-rich conditions, but is also stressful owing to frequent antibiotic therapy and the host immune response. *Pseudomonas aeruginosa* is commonly isolated from the airways of patients with CF, where it most often establishes chronic infections that usually persist for the rest of the lives of the patients. This bacterium is a major cause of mortality and morbidity and has therefore been studied intensely. Here, we discuss how *P. aeruginosa* evolves from a state of early, recurrent intermittent colonization of the airways of patients with CF to a chronic infection state, and how this process offers opportunities to study bacterial evolution in natural environments. We believe that such studies are valuable not only for our understanding of bacterial evolution but also for the future development of new therapeutic strategies to treat severe chronic infections.

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Cystic fibrosis (CF) is a recessively inherited disorder caused by the presence of one of more than 1,500 possible mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, with an incidence of clinical disease of 1 in 2,500 live births[1]. The mutations lead to the malfunction or loss-of-function of CFTR, a cyclic AMP-regulated chloride ion channel, resulting in defective chloride ion transport across epithelial cell surfaces[2]. This decreases the volume of the periciliary fluid in the lower respiratory tract, which in turn interferes with the mucociliary clearance of inhaled microorganisms[2] (FIG. 1). This impairment in the non-inflammatory defences of the respiratory tract causes early recruitment of inflammatory defence mediators, including polymorphonuclear leukocytes (PMNs) and antibodies[3–5]. If not treated, most patients with CF die at a young age owing to airway infections, and in 1974 the international median age at death for these patients was 8 years. Intensive treatment with antibiotics has extended the mean expected lifetime dramatically, however, and patients born in the 2000s are likely to live to a median age of 40 years[6].

In humans, the airways represent a highly compartmentalized environment with distinct habitats such as the paranasal sinuses and the conductive and respiratory zones of the respiratory tree, which are distinguished by different environmental characteristics (FIG. 1). The paranasal sinuses are located in the upper part of the respiratory tract, whereas the conductive and respiratory zones are located in the lower airways. The conductive zone refers mainly to the bronchi, where the airway mucus is produced. This viscous mucus layer is the major reservoir for bacterial reproduction, and it has a heterogeneous distribution and fluctuating levels of antibiotics, nutrients and oxygen, as well as being prone to osmotic and oxidative stresses. The paranasal sinuses also contain thick mucus that can function as a site for bacterial growth. The respiratory zone comprises the respiratory bronchioles and the alveoli, where the presence of bacteria is rare and often linked to substantial lung destruction[7,8]. The environmental factors involved in bacterial adaptation to the lower airway were recently reviewed in detail[9]. In the upper airway, partially or fully concealed sinus cavities have less airflow and less exposure to antibiotics and host immune cells than the lower airways[10,11]. In infected airways in patients with CF, microhabitats can develop owing to local differences in the inflammatory reaction between the different focal areas of infection and also as a result of the competing activities of the many co-colonizing microbial populations.

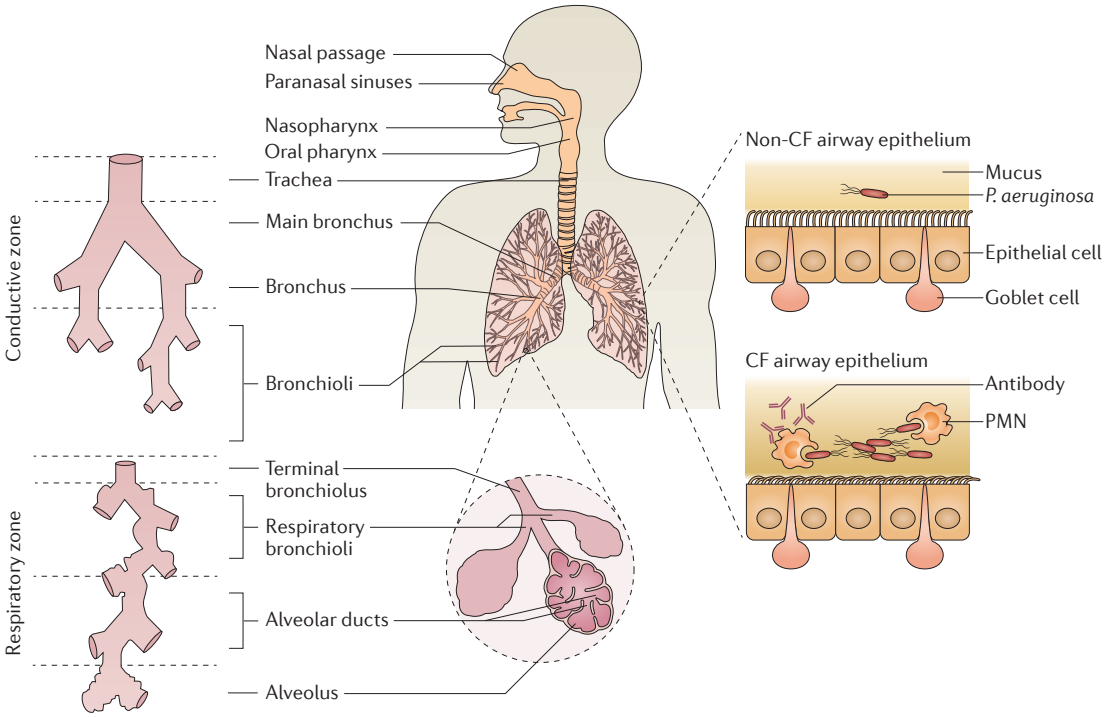

Figure 1 | **Defects in mucociliary clearance of the CF airway creates opportunities for microbial colonization.** The human respiratory system contains different anatomical regions, including the paranasal sinuses, the tracheobronchial region (the conductive zone) and the alveolar region (the respiratory zone). A mucus layer is produced by goblet cells and submucosal glands in the conductive zone and sinuses. In individuals without cystic fibrosis (CF), the cilia of the epithelial cells in the upper airway efficiently remove particles or microbial cells that are trapped in the thin fluidic mucus (a process termed mucociliary clearance). In patients with CF, the cilia cannot clear the viscous and dehydrated mucus layer efficiently, which results in colonization by bacteria such as *Pseudomonas aeruginosa*. The immune responses mediated by polymorphonuclear leukocytes (PMNs) and antibodies lead to scarring of lung tissue and impairment of lung function. The airway mucus is a major reservoir for *P. aeruginosa* growth, and other niches for the bacterium include the paranasal sinuses and the alveoli. Figure is modified, with permission, from REF. 115 © (2008) Annual Reviews.

The microbial flora of the respiratory tract in patients with CF represents a complex and diverse ecosystem in which multispecies communities coexist[12]. The majority of studies of CF-associated pathogens have focused on three major bacterial species, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenzae*, and on the *Burkholderia cepacia* complex species group. Patients with CF have a much higher frequency of *S. aureus* and *P. aeruginosa* infections than most other patient groups and healthy individuals[13]. In spite of the inflammatory response and intensive antibiotic therapy, most infections caused by *P. aeruginosa* persist and become chronic in patients with CF, eventually leading to respiratory failure (FIG. 2a,b) and lung transplantation or death[14]. Studies show that 60–70% of patients with CF are infected by the bacterium in the respiratory tract by the age of 20 (FIG. 2a), but the specific mode of acquisition is unknown[15]. Genetic analysis has shown that most of the initial colonizing strains are unique and originate from unidentified environmental reservoirs[16]. However, transmission of strains among patients can occur, dependent on the frequency of meetings between patients. Chronic airway infection with *P. aeruginosa* is usually preceded by a period of recurrent, intermittent colonization of the airway[17] (FIG. 2c). In this phase, which can last from birth until the patient acquires a chronic infection (usually occurring in their late twenties or early thirties), the infections can be effectively combated with aggressive antibiotic therapy, and this can substantially delay the onset of subsequent chronic infection[5]. The patient is often re-infected at later time points with other *P. aeruginosa* strains of different genotypes, but in approximately 25% of cases re-colonization occurs with the same genotype[18–20]. The source of the re-colonization could be either a persistent environmental source or an undetected reservoir in the patient[21]. As discussed below, the paranasal sinuses potentially constitute one such protected niche from which *P. aeruginosa* can re-colonize the lungs.

For unknown reasons, this intermittent colonization phase, which can last for years, sooner or later transitions into a chronic infection[16]. Chronic infection is characterized by continuous growth of *P. aeruginosa* in airway secretions and by the development of *P.* aeruginosa-specific antibodies (FIG. 2d); it is also correlated with a higher degree of inflammation, a higher number of neutrophils and a greater amount of released serine proteinases than are found in intermittently colonized individuals. Together, these factors cause increased lung obstruction and destruction[5,22–24]. The continuous presence of

Periciliary fluid
The layer of fluid that is in contact with the surface epithelial cells in the airway.

Polymorphonuclear leukocytes
A class of white blood cells that includes neutrophils, eosinophils and basophils, and is characterized by the possession of multilobed nuclei.

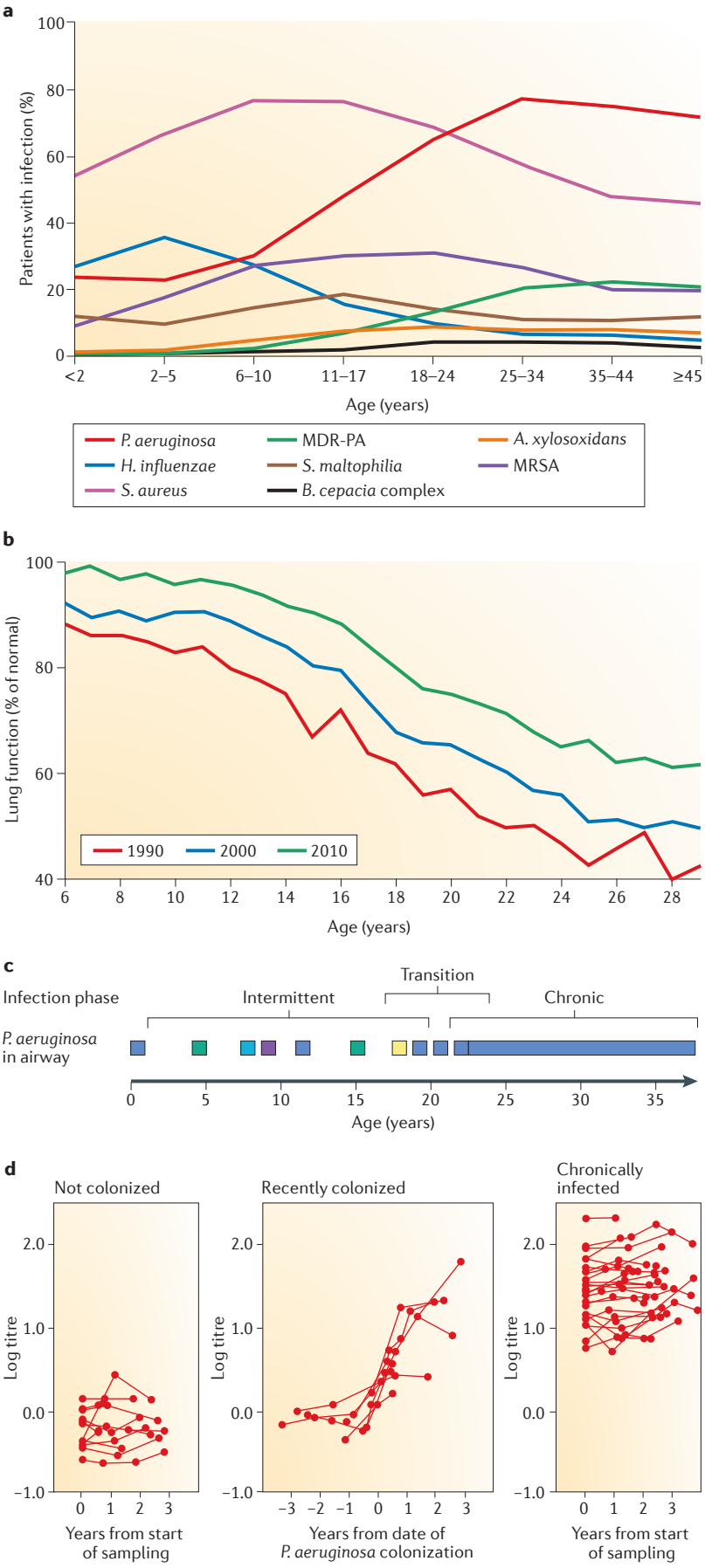

Figure 2 | **Progression of *P. aeruginosa* infections in patients with CF.** **a** | Prevalence of several common human respiratory pathogens in patients with cystic fibrosis (CF) as a function of age. *Pseudomonas aeruginosa* is the most frequently found pathogen in adults. **b** | Decreasing lung function as a function of age in patients with CF. Lung function is measured as the FEV_1 (forced expired volume in 1 second) of patients with CF expressed as a percentage of the FEV_1 of healthy individuals. **c** | Schematic representation of the progression of *P. aeruginosa* infection in a hypothetical patient with CF. Different colours represent phylogenetically independent *P. aeruginosa* clones. Intermittent colonization can be eradicated, and the patients can be negative for *P. aeruginosa* for up to several years. The process is repeated until a chronic infection is established. **d** | Development of *P. aeruginosa*-specific antibodies over time. Sequential log antibody titres are shown, grouped according to the status of colonization with *P. aeruginosa*. Each line represents a single patient. Parts **a** and **b** are reproduced from REF. 6. Part **d** is reproduced, with permission, from REF. 116 © (1991) Wiley and Sons. *A. xylosoxidans*, *Achromobacter xylosoxidans*; *B. cepacia*, *Burkholderia cepacia*; *H. influenzae*, *Haemophilus influenzae*; MDR-PA, multidrug-resistant *P. aeruginosa*; MRSA, methicillin-resistant *S. aureus*; *S. aureus*, *Staphylococcus aureus*; *S. maltophilia*, *Stenotrophomonas maltophilia*.

P. aeruginosa in the chronically infected lung leads to immune complex-mediated chronic inflammation, which is dominated by PMNs and is a major cause of lung tissue damage and decreased lung function, in addition to the damage that is actively caused by the bacteria[25]. In this Review, we discuss how chronic *P. aeruginosa* infections in the airways of patients with CF offer opportunities to study bacterial evolution in natural environments, which are characterized by complexity, spatial structure and dynamic changes over time. Such studies will be valuable for our understanding of bacterial evolution. In addition, such investigations will provide new information that is relevant for long-term bacterial infections. Despite the apparent success of antibiotic treatment in prolonging the lives of patients with CF, the development of antibiotic resistance and the lack of new drugs make it important to identify alternative targets for treatment. It is our conviction that studies of the progression of infection such as those reviewed here will reveal possibilities for novel therapeutic strategies, not only for CF-associated infections but also for other types of chronic infection.

The CF lung environment

The shift that occurs when *P. aeruginosa* moves from the environment into the CF airway involves both nutritional and physicochemical changes for the bacterium, which must survive and adapt to highly stressful conditions that have fundamental effects on its subsequent evolution. The immune system and the constant presence of antibiotics are challenges that must be met effectively before colonization can develop into chronic infection. In addition, competition from other resident microorganisms and the osmotic stress resulting from the high viscosity of the mucus are important factors that

might influence adaptation. Another challenge is that the CF airway changes over time as the infection progresses. For example, the host response to the infecting bacteria leads to airway inflammation and to extensive and irreversible structural changes to the airway[26].

When *P. aeruginosa* encounters stressful conditions such as antibiotics or oxidative stress, a fundamental transition in the gene expression profile occurs. Many of the changes are specific for the particular stress, but there is also a common theme that is shared between the responses to several different antibiotics and to environmental stresses such as osmotic shock and magnesium starvation[27–32]. The switch in gene expression in *P. aeruginosa* is largely governed by the *algU* (also known as *algT*) regulon, which is activated under conditions of cell envelope stress and leads to a coordinated downregulation of central metabolism, motility and virulence, and a concurrent upregulation of genes affecting membrane permeability and efflux[27–32] (FIG. 3). Interestingly, similar genetically determined changes in gene expression are observed when comparing early-colonizing CF-associated strains with those isolated from chronically infected patients with CF[33–35]. Many examples of parallel evolution in CF-associated *P. aeruginosa* isolates have been associated with the selective stress conditions that prevail in the airways, and below we summarize some of these stress conditions to provide the context for a discussion about evolutionary trajectories as they have been documented in the literature.

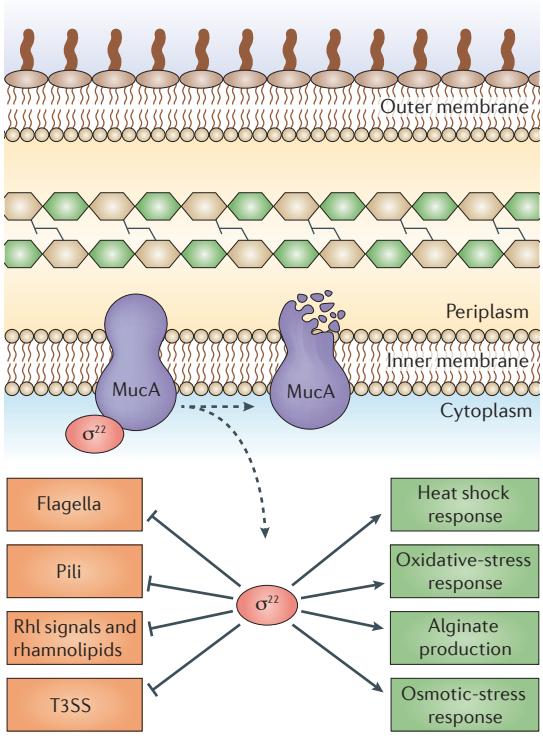

Figure 3 | **Regulation network of MucA–σ22.** The function of the RNA polymerase σ-factor σ22 (encoded by *algU*) is antagonized through protein–protein binding by the anti-σ-factor MucA. Mucoid *Pseudomonas aeruginosa* isolates (which overproduce alginate) from patients with cystic fibrosis most often carry knockout mutations in *mucA*, leaving σ22 free to activate transcription of many genes, such as those involved in alginate production and the responses to heat shock, osmotic stress and oxidative stress[34,117]. σ22 also negatively regulates several virulence factors, including flagella, pili, the type III secretion system (T3SS) and Rhl quorum sensing signals, as well as rhamnolipids that are controlled by Rhl[32,34].

Oxidative and nitrosative stress. The production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) by the host imposes oxidative and nitrosative stresses, respectively, on colonizing bacteria. These molecules are primarily released by PMNs as part of the inflammatory response to infection[36]. In addition to the production of ROS and RNI, patients with CF have impaired protection against antioxidants owing to malabsorption of dietary antioxidants in the gut and the inability of cells harbouring the mutant CFTR protein to efflux glutathione, the most abundant cellular antioxidant, into the extracellular milieu[37,38]. The imbalance between high ROS production and impaired antioxidant protection leads to oxidative stress in the CF airway[39]. When the production of ROS and/or RNI overwhelms the capacity of the bacterial cell to remove such molecules, damage to DNA, lipids and proteins occurs. Both oxidative and nitrosative stress conditions prevail in the thick mucus inside the conductive airway, where activated PMNs liberate ROS and RNI and *P. aeruginosa* grows microaerobically or anaerobically owing to oxygen consumption by PMNs[40,41]. *P. aeruginosa* encounters several sources of oxidative and nitrosative stress during infection of the CF airway, and this can increase bacterial mutation rates (as a result of the increased DNA damage) and select for variants that are able to survive in this environment.

Antibiotic tolerance and resistance. Antibiotics have a fundamental role in the development of CF-associated *P. aeruginosa* lung infections. Before the present-day intensive antibiotic treatment was introduced, patients with CF succumbed to severe *S. aureus* pulmonary infections, and it was not until effective antistaphylococcal therapies were implemented that *P. aeruginosa* was recognized as an important CF-associated pathogen. Therefore, lung infection by *P. aeruginosa* is greatly influenced by the use of antibiotics that generate colonization opportunities in the CF lung environment[42]. Patients with CF are exposed to large amounts of antibiotics during their lives[43], so antimicrobial resistance is common in the bacteria that colonize these individuals; it is likely, therefore, that antibiotics and antibiotic resistance also fundamentally influence the adaptation of *P. aeruginosa* in the CF airway.

Because of the heavy use of antibiotics in CF clinics, *P. aeruginosa* uses a wide variety of different resistance mechanisms to survive[44]. Resistance to all clinically relevant classes of antibiotics is routinely exhibited by *P. aeruginosa* isolates from patients with CF; in these isolates, chromosomally encoded mechanisms of resistance are most frequent, but imported genes encoding

drug-inactivating enzymes also have a role in resistance to β-lactams and aminoglycosides[45–47]. Given the clonal nature of most CF-associated *P. aeruginosa* infections and the apparently infrequent transmission between patients, it would seem that *de novo* evolution of antibiotic resistance in individual patients is common[35,48]. It is clear from several genomic studies that continuous treatment with antibiotics is a major factor in *P. aeruginosa* evolution, shaping the adaptation process by creating antibiotic-resistant lineages of *P. aeruginosa* that expand under the selective pressure of antimicrobial therapy[35,48]. Antibiotic resistance-associated genes are among the most frequently mutated genes during the adaptation process in the CF airway, and the specific genes that are mutated reflect the individual treatment history of the patient from whom the bacterium has been isolated[35,48].

Cell envelope stress. One of the most striking features of *P. aeruginosa* adaptation to the airways of patients with CF is the frequent conversion to a mucoid phenotype owing to excessive and constitutive production of the extracellular polysaccharide alginate, which forms a glycocalyx that covers the surface of the bacterium. Overexpression of alginate is often interpreted as a sign of biofilm development in the CF airway. Alginate production seems to be part of the general envelope stress response of several bacterial species, protecting them from adverse environmental stresses such as desiccation and hydrophobic agents[49]. Alginate protects *P. aeruginosa* from the consequences of inflammation by inhibiting complement activation and decreasing phagocytosis by neutrophils and macrophages[50–52], as well as by sequestering the free radicals that are released from these cells[53]. High levels of alginate have been measured in the sputum of patients with CF who are chronically infected with *P. aeruginosa*[54], and an increase in the antibody response to alginate has been linked with a poorer prognosis for patients with CF[55,56].

The most common mutations responsible for the mucoid conversion are found in *mucA*, which encodes an inner-membrane-associated anti-σ-factor[57,58] (FIG. 3). MucA normally limits the expression of the *algD* operon, which encodes the enzymes required for alginate synthesis, by sequestering the alternative RNA polymerase σ-factor σ^{22}, encoded by *algU*[59–63]. In addition to the *algD* cluster, σ^{22} regulates a large number of stress response and virulence-associated genes and is involved, directly and indirectly, in the regulation of virulence and motility in *P. aeruginosa*[42,55,59,64,65].

This suggests that the importance of the *mucA* mutations goes beyond the conversion to a mucoid phenotype. In fact, the primary selective advantage of the *mucA* mutations might be activation of the cellular envelope stress response, and the overproduction of alginate might be a secondary consequence of the mutations. This is in agreement with the observation that mucoid *P. aeruginosa* is most often found in co-infections with non-mucoid revertants that still harbour a *mucA* mutation, and in fact some patients with CF are chronically infected by only non-mucoid *P. aeruginosa*. As mucoid and non-mucoid *P. aeruginosa* isolates from chronically infected patients with CF often have mutations in *mucA*, and non-mucoid isolates from the chronic stage of the infection are frequently descendants of the original mucoid strain that infected the patient, it seems that *mucA* is the repeated target for selective mutations during adaptation to life in the CF lung[21,34,66].

In summary, many of the characteristic phenotypes of CF-associated *P. aeruginosa* isolates are associated with colonization in a stressful environment — the CF airway — in which the inflammatory response of the host and the often constant presence of antibiotics select for protective mutations. Interestingly, several of the mutations that are frequently identified in these isolates affect activities associated with increased protection from stress conditions[35].

Colonization and chronic infection in CF airways

Organisms that colonize rapidly changing environments such as the CF airway are exposed to particular adaptive problems. They can evolve to become either specialists in a particular niche or generalists capable of surviving and competing effectively in many environments. Spatially structured environments are thought to enhance diversification, specialization and fitness trade-offs[67,68], and *P. aeruginosa* strains isolated from patients with CF often exhibit remarkable phenotypic diversity, as documented by the appearance of many different colony morphology variants[55,69,70], the development of hypermutability[71] and the range of antimicrobial susceptibility in these strains. It has been proposed that this diversity is associated with specializing adaptation to the different compartments in the CF airway[72].

There is a close association between bacteria isolated from the upper and lower airways of patients with CF, as the paranasal sinuses of these individuals are often colonized with CF-associated pathogenic Gram-negative bacteria[70,73,74]. Antibiotic penetration, and hence the achievement of therapeutic levels, is less efficient in the sinus cavities than in the lungs[75], and chronic rhinosinusitis has frequently been observed in patients with CF[76]. Patients who are intermittently or chronically infected with *P. aeruginosa* show close agreement between the bacteria residing in their lungs and their sinuses. Two studies that carried out simultaneous nasal lavage and sputum sampling found that *P. aeruginosa* and *S. aureus* strain pairs were genotypically identical in 95% and 86% of tested individuals with CF, respectively, and there appeared to have been cross-infection between the two compartments[77,78]. After intensive functional endoscopic sinus surgery (FESS) in 78 patients with CF, it was found that 18 out of 21 patients with a chronic *P. aeruginosa* infection were infected in both the lungs and the sinuses, and all 18 had identical *P. aeruginosa* genotypes in the two locations; moreover, 22 out of 31 intermittently colonized patients had *P. aeruginosa* in both the lungs and sinuses, and 20 of these had identical isolates in both locations[74]. It has furthermore been shown that several of the mutations characteristic of chronically infecting *P. aeruginosa* strains can also be found in isolates from the sinuses in patients who do not yet show any sign of chronic lung infection[70]. Colonization of the sinuses

Envelope stress response
A signal transduction pathway that senses and responds to extracytoplasmic stress.

Complement
Part of the innate immune response. The complement system comprises more than 25 proteins that recognize foreign objects and target them for destruction or phagocytosis.

Anti-σ-factor
A negative transcriptional regulator that binds to a target RNA polymerase σ-factor to prevent its activity.

Hypermutability
A mutation rate that is high compared with the average mutation rate for the species.

Chronic rhinosinusitis
In adults: inflammation of the nose and the paranasal sinuses, characterized by two or more symptoms, one of which should be nasal blockage, obstruction, congestion or discharge (anterior or posterior nasal drip). The other symptoms are one or more of the following: facial pain or pressure; a reduction or loss of smell; a cough; endoscopic signs of nasal polyps; mucopurulent discharge (primarily from the middle meatus); oedema or mucosal obstruction (primarily in the middle meatus); and CT changes.

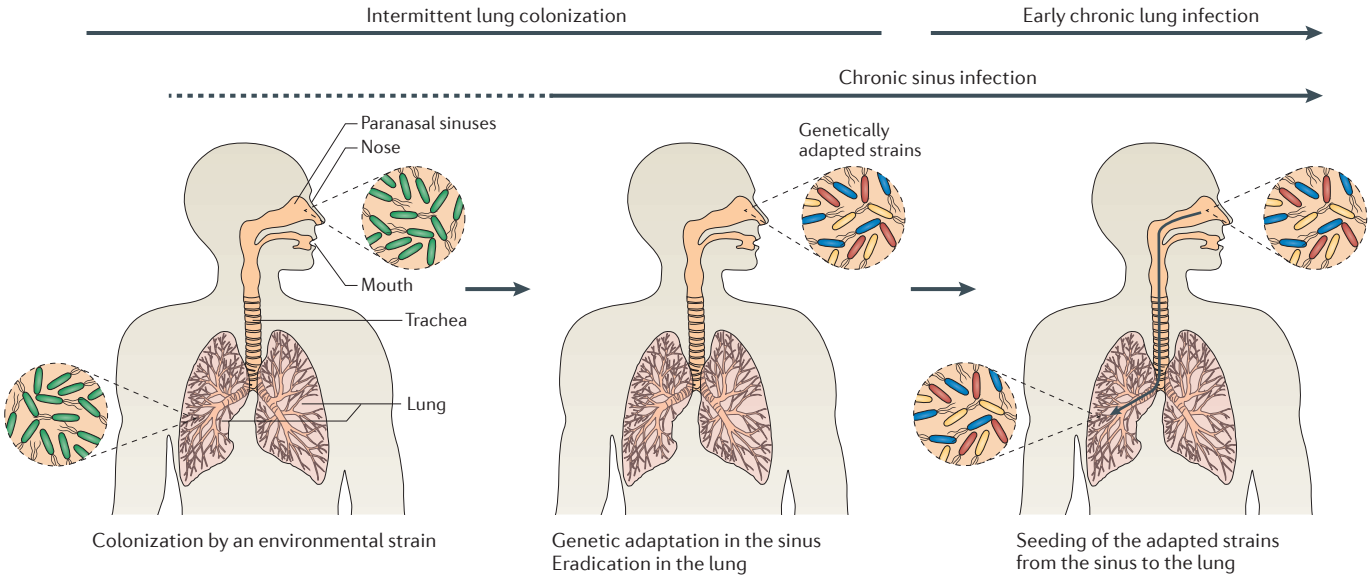

Figure 4 | **A model for the typical course of *P. aeruginosa* colonization and infection in the CF airway.** *Pseudomonas aeruginosa* most often invades the airway of patients with cystic fibrosis (CF) from the environment. Colonization can occur both in the paranasal sinuses and in the lungs. Compared to in the lungs, the immune response and antibiotic-mediated stress in the sinuses are less severe, providing a protected niche for bacterial colonization. During waves of antibiotic treatment and host immune responses, *P. aeruginosa* can be eradicated from the lungs. Subsequently, the lungs can be recolonized by the same *P. aeruginosa* genotype, which has survived the immune and antibiotic attacks in the sinuses. These cycles of eradication and reinfection constitute a period of intermittent colonization. During colonization of the sinuses, adaptive mutations have been documented to occur, and these gradually increase the fitness of the bacteria. Thus, it appears that the chronic nature of an infection might be established initially in the paranasal sinuses, after which the adapted strains migrate to the lower airway, resulting in chronic infection of the lungs[70].

before lower-airway infection could thus facilitate the evolution of strains that are more adapted to the stressful lung environment (FIG. 4). To elucidate whether CF-associated pathogens can potentially be eradicated with FESS and postoperative antibiotic treatment, we need to undertake studies using longer follow-up times and larger cohorts with the possibility of randomizing patients to different treatment groups.

In the Copenhagen CF Centre (Copenhagen University Hospital, Denmark), chronic *P. aeruginosa* infection has been defined as the continued presence of the bacterium over a period of at least 6 months and/or the presence of two or more precipitating antibodies against *P. aeruginosa* in the serum. This clinical definition is also important in many other CF clinics for the design of therapeutic strategies, but it might not cover the colonization and infection events that occur in the CF airway, and the adaptive transition of the bacterium to the chronic infection state might have occurred long before the clinical signs are detectable. As an example, in one study it was found that in some patients the same *P. aeruginosa* genotype had been colonizing the lungs intermittently for up to 6 years before the patient underwent FESS, although every time the bacterium was cultured from the patient's sputum it had apparently been subsequently eradicated by antibiotic therapy. If the same clone of *P. aeruginosa* persists for such long periods of time, it should be considered chronic despite the lack of precipitating antibodies. There may therefore be a need to modify the operational definition of chronic infection[74].

Evolution of *P. aeruginosa* in chronic infections

Chronic airway infections in patients with CF are associated with genetic and phenotypic changes in the infecting bacteria. Thus, the phenotypic characteristics of a *P. aeruginosa* isolate from a chronically infected patient with CF are often remarkably different from the characteristics displayed by the clonal isolate that initiated the infection in the patient. Many of these phenotypic changes are observed repeatedly in isolates sampled from different patients and clinical settings. For example, these isolates are typically characterized by their slow growth, antibiotic resistance, lack of motility, loss of quorum sensing, changed cell envelope and overproduction of alginate. Some studies have used transcriptomic profiling of longitudinal clonal isolates from different patients with CF to identify common changes in the 'expression signature' of each strain. One such study identified a set of 24 genes that exhibited parallel changes over time in three patients with CF[33]. The repeated recurrence of particular characteristics (phenotypes and gene expression traits) suggests that they are the result of parallel evolution of the bacteria within the hosts and that they are advantageous (that is, adaptive) for bacterial growth and survival *in vivo*. However, it is important to note that in spite of the apparent parallelism in the phenotypic changes among clinical isolates, there is a high level of phenotypic diversity within an individual patient, which is often observed as different colony morphologies[55,69,70]. When multiple isolates from the same sputum sample are analysed, such phenotypic

diversity is also common with respect to several of the phenotypes mentioned above. This diversity of the bacterial populations in the CF airway has been interpreted as an indication of the presence of cheaters, which benefit from the production of 'public goods' (such as quorum sensing signals or siderophores)[79]. Importantly, the presence of hypermutator strains in the bacterial populations of the CF airway is most often associated with the parallel occurrence of different subpopulations with different phenotypic traits. Thus, generalizing conclusions concerning the population structure of *P. aeruginosa* in the CF airway should be considered with caution, as they are often based on investigations of single bacterial isolates from samples taken at different time points.

Although the appearance of parallel 'chronic, CF-evolved phenotypes' might indicate that there is a limited number of adaptive peaks in the fitness landscape, the actual evolutionary route towards this common phenotypic profile and the degree of route sharing among chronically infecting clones of different genotypes sampled from different clinics and patients are still not well understood. Recent tracking of genomic evolution in sequential isolates from individual patients has begun to address these issues. In one ground-breaking longitudinal study, researchers first sequenced the genomes of a pair of *P. aeruginosa* isolates sampled from a patient with CF, the second isolate having been obtained 90 months after the first; they then identified the mutational changes that had accumulated in the later descendant compared with the early isolate[48]. By tracking these mutations in a collection of *P. aeruginosa* isolates from 29 other patients with CF, it was possible to identify a set of genes that had repeatedly acquired non-synonymous mutations in the descendants. This confirmed a number of previous findings of similar genetic changes in CF-associated *P. aeruginosa* isolates from many different patients and clinics. Examples of such recurring genetic changes include mutations that inactivate MucA and thus result in overproduction of alginate and conversion to the mucoid phenotype, as discussed above[57]. Loss-of-function mutations are also frequently found in *lasR*, which encodes a transcriptional regulator of quorum sensing[80]. Inactivation of LasR decreases the expression of many important virulence genes and confers a growth advantage in the presence of certain amino acids[81].

In a recent study, the genomic evolution of two additional *P. aeruginosa* lineages was examined[82]. In one lineage, which belongs to the PA14 clonal complex, only 15 SNPs accumulated over a period of ~15 years. The other lineage remained unchanged during the first 3 years of infection, after which hypermutator variants appeared, and close to 1,000 mutations accumulated in the lineage during the following 15 years[82]. Hypermutator variants also appeared in the longitudinal study[48]. Although mutation rates clearly influence the speed of adaptive changes, other factors — such as the particular genetic configuration of the original infecting lineage, as well as epistatic interactions among the mutations that accumulate — might restrain certain evolutionary routes while opening up other pathways for adaptation[83–85]. In this context, it is interesting that mutations in global regulatory genes are observed frequently in CF-associated *P. aeruginosa* isolates[35,48,80]. It is possible that the characteristics of the specific mutations and their specific combinations result in lineages that are particularly successful in the CF environment.

Clonal complex
A group of bacterial isolates, as derived from multilocus sequence typing (MLST) analysis. A clonal complex usually includes isolates that differ from each other at only one of the seven loci analysed by MLST.

An adapted lineage of *P. aeruginosa*

Chronic infection of the CF airway by *P. aeruginosa* is a valuable scenario with which to study bacterial evolution in a complex natural environment. These infections are most often clonal, and a single *P. aeruginosa* lineage can colonize the lungs of an individual patient for years and even decades[86–88]. In addition, regular sampling of the infecting bacteria is possible from, for example, sputum produced by the patients during normal care. These features make it possible to sample, store and analyse sequential clonal isolates from the CF lung environment, and with the recent developments in DNA sequencing and other genome- or organism-wide analytical tools such as transcriptomic and metabolomic techniques, it is now possible to produce a detailed characterization and time-resolved map of the evolutionary trajectories of the infecting bacteria. As described above, in recent years several research groups have exploited such strain collections to obtain information about the evolution of *P. aeruginosa* and other bacteria that persist in the CF airway[89–94]. Most of these studies were recently reviewed in a paper focusing on adaptive evolution directed by hypermutators and on alterations of general virulence phenotypes and physiological traits[93]. Here, we focus mainly on the genetic changes that seem to drive the infecting bacteria towards peaks in the fitness landscape of the CF airway.

The *P. aeruginosa* strain collection at the Copenhagen CF Centre represents nearly 40 years of 'infection history' from a large number of patients, which makes it possible to design longitudinal investigations of evolutionary trajectories for different bacterial clones in different patients on a timescale that covers decades of bacterial growth. What further makes this historic strain collection ideal for evolutionary studies is the observation that among the many different strains of *P. aeruginosa* present in the collection, two clone types have been encountered repeatedly because of frequent inter-patient transmission[21]. In fact, each of these two dominant transmissible clone types, *P. aeruginosa* DK1 (formerly the r genotype) and *P. aeruginosa* DK2 (formerly the b genotype), has been isolated since the start of the sampling programme in 1973, each from approximately 40 patients. Although efficient cohort isolation in the clinic has prevented further spread of these strains, the fact that these two distinct clones, which independently evolved into successful colonizers of the CF airway, are dominant in a large group of chronically infected patients provides opportunities to systematically study the evolutionary dynamics of these bacteria during infection in ways that are often only possible with bacterial populations evolving in defined laboratory settings.

From the estimated generation times of *P. aeruginosa* DK1 in the patient airway, it was concluded that

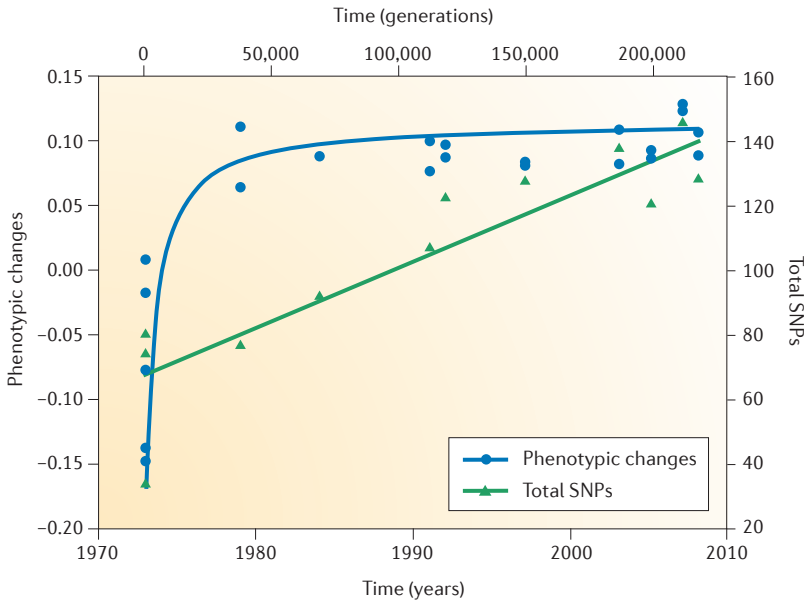

Figure 5 | **Rates of mutation accumulation and phenotypic changes in CF-associated isolates of *P. aeruginosa*.** The evolutionary dynamics of the transmissible *Pseudomonas aeruginosa* DK2 lineage was investigated by characterizing 12 isolates sampled between 1973 and 2008 (corresponding to approximately 200,000 bacterial generations) from six patients with cystic fibrosis (CF)[35]. For each isolate, SNP accumulation relative to the last common ancestor of the DK2 lineage was determined from whole-genome sequencing and shows that the mutations accumulated in a linear manner. The phenotypic changes of the lineage over time were measured by transcriptomic profiling and analysis of the catabolic activities of all the isolates. Phenotypic differences between each isolate were quantified using principle component analysis; see REF. 35 for details. Here, the loadings of the first principle components of the two phenotypic data sets are plotted as a function of time, and they fit a hyperbolic model that exhibits a major shift between 1973 and 1979.

the dominant lineages have persisted in the airways of several patients with CF for approximately 200,000 bacterial generations[35,89]. This is in the range of the number of generations separating present-day humans and chimpanzees from their last common ancestor and represents approximately four times the generation length of the longest running long-term evolution experiment (LTEE) with *Escherichia coli*, which was started by Richard Lenski in 1988 (REF. 95).

The dynamics of evolutionary adaptation and genomic changes. Bacterial populations that are introduced experimentally into new environments in the laboratory typically have an initial period of rapid adaptation, after which the rate of adaptation decelerates over time. Such nonlinear dynamics indicate that, after bacteria are placed in a new environment, early mutations have larger fitness effects, when the population is far from an adaptive peak in the fitness landscape, whereas substitutions that are acquired later have smaller effects, and eventually the magnitude of the effects plateaus. Although *in vitro* experiments have provided measurements for such dynamics of microbial adaptation in defined settings, little is known about the evolutionary dynamics in natural and more complex environments, such as human hosts.

Using the collections of sequential *P. aeruginosa* clonal isolates, it has been possible to map the evolutionary dynamics of *P. aeruginosa* evolving in the CF airway, but only in the case of the Copenhagen CF Centre collection have the details been carefully characterized. Using measurements of changes in cellular traits such as global gene expression profiles and catabolic capacity, the phenotypic-adaptation trajectory of the *P. aeruginosa* DK2 lineage was characterized as this lineage disseminated among a group of patients with CF over a time period of 35 years. Similar to the results from the LTEE laboratory model[96] and experiments with bacteriophages[97,98], the adaptation was found to be nonlinear; the *P. aeruginosa* DK2 lineage underwent an initial period of rapid adaptation on entering the CF airway environment (between 1973 and 1979), followed by a period of 29 years (estimated to correspond to approximately 150,000 bacterial generations) of limited phenotypic change (FIG. 5). Sequencing the genomes of several *P. aeruginosa* DK2 isolates sampled between 1973 and 2008 led to the identification of the mutations that accumulated during this period (FIG. 5). The rate of genomic evolution was found to be nearly constant, with SNPs accumulating in a near linear manner (at a rate of 7.2×10^{-11} SNPs per bp per generation) during the 35 years of colonization of individuals with CF. A total of 180 SNPs separate one of the first isolates, from 1973, and an isolate from another patient sampled 35 years later[35]. Although these findings appear to be similar to the recent findings about the relationship between rates of adaptation and genomic evolution of *E. coli* in the LTEE[95], the similarity is only superficial, as the *P. aeruginosa* str. DK2 lineage exhibited a genomic signature of negative selection (dN/dS<1; in which dN/dS is the ratio of non-synonymous (dN) to synonymous (dS) mutations) in contrast to the continuous accumulation of non-synonymous mutations in the *in vitro E. coli* evolution experiment.

The finding that there was a long period of phenotypic stability and a signal of negative selection in the *P. aeruginosa* str. DK2 population is surprising given that the isolates were sampled from a number of different — although parallel — habitats (the airways of different patients with CF), each of which is composed of multiple and variable niches. The complex and heterogeneous environmental conditions in the CF airway would be expected to generate and maintain population diversity, similarly to bacterial populations evolving in laboratory systems in which artificial environmental heterogeneity (such as spatial structures and community complexity) has been imposed on the evolving population[68,99–101]. The limited diversification observed in the later stages of *P. aeruginosa* str. DK2 evolution is also in contrast to the frequent observations of phenotypic heterogeneity in clonal *P. aeruginosa* populations within the same respiratory specimen[34,81,102]. One possible explanation for the observed phenotypic stability and genomic signature of negative selection would be that the *P. aeruginosa* str. DK2 lineage became positioned on a major adaptive peak in the fitness landscape. As a consequence, most new mutations would not be fixed in the population, as they would result in reduced fitness.

Overall, the emerging picture is that evolution of *P. aeruginosa* str. DK2 developed in two stages. First, there was a period of adaptive evolution following migration of the ancestral *P. aeruginosa* str. DK2 from its natural environment into the host with CF. This early period of evolution might have been dominated by positive selection and adaptive mutations, as noted recently[48]. Second, a period of little adaptive evolution dominated by negative selection followed and has lasted until today. The total evolutionary progression has resulted in a highly successful lineage with the capacity for long-term survival in, and transmission between, several hosts with CF.

Success through global regulatory mutations. Major evolutionary transitions can involve changes in gene regulation[103], and a recurring observation from bacterial evolution experiments in the laboratory is the occurrence of evolutionarily important mutations in global regulatory genes[104–106]. Similar pleiotropic adaptive mutations can also be observed in natural settings[107,108]. From the genomic data described above for *P. aeruginosa* str. DK2, it appears that the lineage acquired a set of mutations in three global regulators, resulting in large-scale changes in both the gene expression profile and the catabolic capacity of the lineage. These mutations were acquired at early time points in the assessed period (pre-1979), and one can speculate that the characteristics of the specific mutations (in *mucA*, *lasR* and *rpoN* (encoding RNA polymerase σ-factor σ^{54})) and their particular combination — most likely in conjunction with mutations in genes affecting susceptibility to various antibiotics — resulted in a lineage that was highly successful in the CF airway environment and had the capacity to be transmitted among individuals with CF. It is intriguing that evolutionary trajectories can to some extent be determined by the occurrence, and perhaps also the sequence, of mutations in global regulators, and it will be interesting to see, in future investigations, whether other combinations of regulatory mutations can also direct evolution towards increased adaptive performance. Another question for future research is whether the evolutionary route outlined here for the *P. aeruginosa* str. DK2 lineage has been shared by other chronically infecting *P. aeruginosa* clones or whether adaptation has been accomplished through different routes.

It is important to stress that the analysis of evolutionary changes offered here is focused on single-nucleotide substitutions. However, other genetic changes (such as insertions and deletions; mobilization of transposons, phages and genomic islands; and other types of genomic rearrangement) have been documented in populations of *P. aeruginosa* residing in the CF airway, but these topics lie outside the scope of this Review[34,109,110].

Hypotheses for future investigations

Experimental evolution is increasingly being used as an investigative method[111]. The knowledge gained from well-controlled and well-conducted bacterial experiments has provided fundamental information about the evolutionary processes in defined environments, and the conclusions drawn from such scenarios are now available for comparison with those drawn from the evolution of natural populations in more complex settings. Here, we have reviewed one example of natural evolution — adaptation of the bacterium *P. aeruginosa* to the environment in the airway of patients with CF.

We think that there are five key hypotheses that can be drawn from this work which are relevant for future investigations. First, *P. aeruginosa* and other microorganisms colonizing the CF airway are confronted with a highly selective and heterogeneous environment characterized by various types of stress. Factors such as antibiotics, the host immune response, and oxidative and osmotic stress are all important evolutionary forces that select for genetic variants with an improved ability to survive and proliferate in the CF-associated environment. Sequencing the genomes of such ecologically successful variants sampled from chronic infections in different patients with CF and at different clinics has shown that these variants often share mutations in genes associated with increased stress protection. Second, it seems that the sinuses of patients with CF might act as protected reservoirs for the adaptation of *P. aeruginosa* to the lung and for later seeding of the rest of the airway[70]. The realization that the sinuses might play an important part in the lung adaptation process opens up the possibility of alternative therapeutic interventions that focus on the microbiology of this region of the airway and that could potentially delay the onset of the chronic stages of infection. Third, only a limited number of mutations in important global regulators might be necessary to transform an opportunistic pathogen that causes acute infection into a persistent pathogen that can spread to susceptible hosts. This illustrates how only a few genetic changes can have fundamental effects on the ecology and lifestyle of microorganisms. Fourth, the adaptive changes in the bacterial population that lead to the chronic stage might occur long before the clinical criteria of chronic infection are met. Fifth, the evolutionary dynamics of *P. aeruginosa* during adaptation to the CF airway resembles the dynamics documented from *in vitro* experimental evolution[35,96–98]. Despite the stressful and fluctuating environment of the CF airway, it seems that at least some adapting populations of *P. aeruginosa* manage to reach a state of fitness characterized by limited genomic change and under which negative selection is the predominant force shaping evolution.

The hypothesis outlined above should take into account the complexity of the CF airway microbiome. There are several reports documenting the high level of species diversity in the CF airway[12,112–114], and it is quite possible that some of the evolutionary processes observed for *P. aeruginosa* are influenced to some extent by the presence of other members of the microbial community. Future investigations will shed light on this very interesting aspect of adaptation. In addition, this feature of the airway ecosystem adds further relevance to this model system as representative of natural ecosystems. It is also important to emphasize that comparisons of the specific adaptive processes occurring in

patients attending different clinics might be problematic owing to there being different treatment scenarios offered by the various clinics and because of the different genetic backgrounds of the respective infecting *P. aeruginosa* strains.

The application of sequencing and transcriptomic technologies has greatly improved our understanding of the evolutionary events at both the nucleotide and gene expression levels in *P. aeruginosa* during infections of patients with CF, and such studies have already provided important information to inform future therapeutic strategies. Genes that are controlled by some of the important regulators might constitute novel antimicrobial targets, and trade-off consequences from mutations in the regulatory genes could possibly be exploited as weak defence barriers of the infecting bacteria. Further studies of microbial evolution in CF-associated infections, coupled with novel technologies in areas such as microfluidics, metagenomics and metabolic profiling, will be required to identify biomarkers for the transition to a chronic infection state at an earlier time point, when eradication of the infecting bacterial strain is still possible. If experimental studies of the evolutionary pathways of chronic *P. aeruginosa* infections are connected to evolutionary and ecological theory, the result will hopefully be improved treatment of the infections, directly based on the resulting evolutionary knowledge.

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Competing interests statement
The authors declare no competing financial interests.

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Online summary

- Cystic fibrosis (CF) is caused by a mutation in the CF transmembrane conductance regulator gene, which encodes a cyclic AMP-regulated chloride ion channel. The mutation results in defective ion transport across epithelial cell surfaces in the upper airways, interfering with the clearance of particles and microbial cells that are trapped in the overlying mucus and resulting in a greatly increased susceptibility to bacterial infection. As a consequence, the airways of patients with CF are nearly always infected with many different bacterial species, but *Pseudomonas aeruginosa* infection causes the greatest burden of morbidity and mortality.
- *P. aeruginosa* infection of the airways of patients with CF begins with a period of recurrent, intermittent colonization from the environment or from a protected niche within the patient, such as the paranasal sinuses. These intermittent infections can be effectively controlled with aggressive antibiotic therapy, but eventually a chronic infection develops that can persist for the rest of the patient's lifetime.
- Chronic infections of the CF airway by *P. aeruginosa* thus provide a valuable opportunity to study bacterial evolution in a complex natural environment. These infections are most often clonal, and regular sampling is possible from samples collected when the patients attend clinics. With the recent developments in DNA sequencing and other genome-wide analysis tools, it is now possible to produce a detailed characterization and time-resolved map of the evolutionary trajectories of the infecting bacteria.
- In the CF lung, *P. aeruginosa* encounters stressful conditions, including oxidative, nitrosative and cell envelope stress, and antibiotics. The switches in *P. aeruginosa* gene expression that occur in response to these stresses and to antibiotics have been well characterized, and the global regulators responsible have been identified.
- Recent tracking of genomic evolution in sequential *P. aeruginosa* isolates from individual patients has identified a set of genes that were repeatedly found to have acquired non-synonymous mutations. These genes include three global regulators that were known from *in vitro* work to be important in *P. aeruginosa* adaptation to the CF lung: *mucA*, which encodes an inner-membrane-associated anti-σ-factor and controls alginate synthesis; *lasR*, which encodes a transcriptional regulator of quorum sensing; and *rpoN*, which encodes an alternative RNA polymerase σ-factor involved in the regulation of virulence gene expression.

ToC blurb