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# Microevolution of *Pseudomonas aeruginosa* in the airways of people with cystic fibrosis

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The chronic infections of cystic fibrosis (CF) airways with *Pseudomonas aeruginosa* are a paradigm of how environmental bacteria can conquer, adapt, and persist in an atypical habitat and successfully evade defense mechanisms and chemotherapy in a susceptible host. The within-host evolution of intraclonal diversity has been examined by whole-genome sequencing, phenotyping, and competitive fitness experiments of serial *P. aeruginosa* isolates collected from CF airways since onset of colonization for a period of up to 40 years. The spectrum of de novo mutations and the adaptation of phenotype and fitness of the bacterial progeny were more influenced by the living conditions in the CF lung than by the clone type of their ancestor and its genetic repertoire.

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## Introduction

Unlike numerous viruses such as the herpesviruses that infect humans early in life and may persist lifelong in latent form, long-term infections of a human host with bacteria are currently confined to a few species, namely *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Pseudomonas aeruginosa*. *H. pylori* and *M. tuberculosis* were already prevalent in humans in prehistoric times, but the chronic airway infections with *P. aeruginosa* in patients with cystic fibrosis (CF) emerged just 60 years ago. Hereunder, we review our current knowledge of the microevolution of *P. aeruginosa* in the CF airways, including key features of *P. aeruginosa*, the CF host, and their interaction within the airways. The focus will be laid upon own work executed within the frame of the Collaborative Research Centre 900 supported by the German Research Foundation (DFG), but moreover, we will summarize general knowledge and important contributions of our peers with emphasis on publications for the period 2018– 2022.

## *Pseudomonas aeruginosa*: lifestyle, pathogenicity, and population biology

*P. aeruginosa* is a metabolically versatile Gram-negative bacterium that is ubiquitously present in inanimate soil and aquatic habitats at low frequency and can colonize the animate surfaces of plants, animals, and humans. The bacterium preferentially thrives and outcompetes other microbes in nutrient-poor aquatic habitats making the hospital environment a favorable niche to take residence [1]. Hospitals accommodate large proportions of vulnerable individuals with reduced defense mechanisms who are prone to nosocomial infection and thus may acquire *P. aeruginosa* from inanimate or animate sources.

*P. aeruginosa* is classified as an opportunistic pathogen that causes a wide range of infections in humans, involving nearly all-body systems, which vary from local to systemic and from self-limiting to life-threatening [2]. The bacterium is equipped with a lowly permeable outer membrane and multiple transport systems, rendering it naturally resistant to many antimicrobial agents. Moreover, *P. aeruginosa* may acquire resistance to nearly all available antimicrobials by genomic mutation or horizontal uptake of resistance determinants [3]. Consequently, the World Health Organization (WHO) has included *P. aeruginosa* into the ESKAPE pathogens that are a global threat because of their capacity to become increasingly resistant to all available antibiotics [4]. Multidrug-resistant (MDR) *P. aeruginosa* strains are more frequently found in intensive care units (ICU) than in non-ICU settings, except for respiratory isolates, which generally have high rates of MDR *P. aeruginosa* in the hospital environment [5]. Recurrent infections of the urogenital tract are frequent in individuals with paraplegia and chronic infections are mainly seen in the respiratory tract of individuals with chronic obstructive pulmonary disease (COPD), bronchiectasis, and CF.

## Cystic fibrosis: inherited susceptibility to airway infections

CF is a severe autosomal-recessive trait caused by mutations in the *CFTR* gene that encodes an epithelial ion channel for the secretion of chloride and bicarbonate into the ducts of all exocrine glands [6]. In CF, the reduced pH of airway surface liquid and the reduced chloride secretion impair bacterial killing by the innate immunity and the detachment of mucus from submucosal gland ducts [7]. Mucus plugging in the ducts predisposes to colonization with opportunistic pathogens, first *Staphylococcus aureus* and later in life *P. aeruginosa*, that initiates a vicious cycle of infection, inflammation, and tissue remodeling, which — if untreated — leads to a continuous decline of lung function and finally to premature death because of respiratory insufficiency [6]. However, during the last 50 years, efficacious therapeutic measures have been developed to contain infection and inflammation [8].

## Clinical epidemiology of the chronic airway infections with *P. aeruginosa* in cystic fibrosis

CF is a moving target for *P. aeruginosa*. In the early 1980s when no therapeutic guidelines existed, the first detection of *P. aeruginosa* in the patient's respiratory secretions was classified as the 'harbinger of death for any patient' [9]. By 2022, the carriage of *P. aeruginosa* is still a substantial comorbidity, but not anymore a risk factor for a poorer prognosis. More the opposite, the elderly CF 'survivors' have a better outcome than their peers if they are chronically colonized with slow-growing, attenuated *P. aeruginosa* [10]. In other words, the course and prognosis of the Pseudomonas infections in CF have been substantially influenced by the temporal changes of treatment modalities and of median survival that improved, for example, in Germany from 9.2 years in 1980 to 54.2 years for the period 2015–2019 [11].

Once *P. aeruginosa* has taken permanent residence in the small conducting CF airways, one can suppress the bacterial load by topical and/or systemic antimicrobial chemotherapy and thereby reduce inflammation and improve lung function, but it is virtually impossible to eradicate the microorganism by antimicrobials [12]. Hence, protocols for the early intervention upon positive *P. aeruginosa* culture have been developed, and inhaled tobramycin or colistin were indeed successful to shift the average onset of chronic airway colonization from school age to early adulthood [12,13]. Meanwhile, highly efficient cystic fibrosis transmembrane conductance regulator (CFTR) modulators, which partially reverse the basic defect, have become available to 90% of people with CF [14,15]. Continuous triple-combination therapy with Elexacaftor/Tezacaftor/ Ivacaftor removed *P. aeruginosa* from chronically colonized CF airways [16]. Current real-world epidemiologic data from the German CF registry indicate that the success rate differs by age group and colonization status between 15% and 50% (Sieber et al., submitted). Thus, by 2022, the lifelong airway infections with *P. aeruginosa* in CF have been transformed to a condition amenable to therapeutic elimination by CFTR modulators.

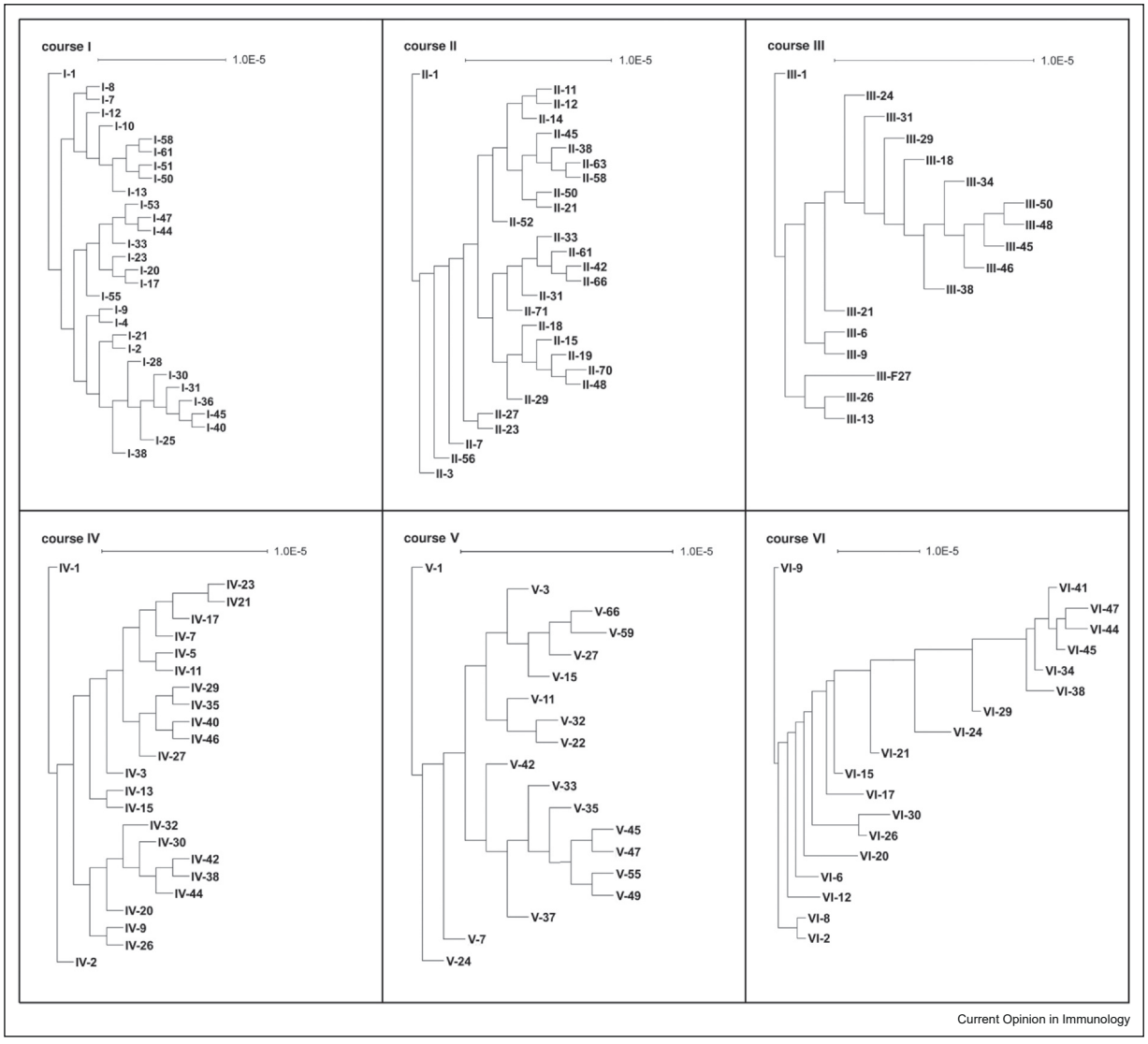
## Microevolution of *P. aeruginosa* genotype in cystic fibrosis airways

Within-host evolution of *P. aeruginosa* was deduced from whole-genome sequencing of serial CF isolates of lineages collected since onset of colonization in patients seen at the CF clinics in Copenhagen and Hannover [17–23] (Figure 1) or whole-genome sequence comparisons of large collections of CF and non-CF isolates [24] (reviews: [25,26]) (Table 1). Non-neutral mutations predominantly emerged in *P. aeruginosa* genes relevant for protection against and communication with signals from the lung environment, that is, antibiotic resistance, cell-wall components, and two-component systems [26]. Within CF lungs, the disease-specific hot spots of mutation are *P. aeruginosa* genes that govern the stringent response (*relA*/*spoT*), modulate the composition of mannuronic and guluronic acid in the exopolysaccharide alginate (*algG*), or modify the core oligosaccharide of the highly immunogenic lipopolysaccharide (LPS) for immune evasion of the host response (*pagL*) [27]. Like in other human infections, loss-of-function mutations in *lasR* perturb or rewire quorum sensing (QS), a three-unit regulatory system that controls the expression of virulence factors and secreted public goods [28,29]. Loss-of-function mutations in *mutS*, *mutL*, or *uvrD* inactivate the mismatch repair system and lead to hypermutable strains [30] that accumulate point mutations [17] and tolerate the insertion of mobile genetic elements [22].

We have collected serial semiannual *P. aeruginosa* isolates from all CF patients regularly seen at the CF clinic, Hannover, who became chronically colonized in their airways between 1982 and 1991. Most patients were chronically carrying the initially acquired clone for ten years or more and were transiently cocolonized for a few months with up to four further clones during the initial 30-year observation period [31]. We examined the genome evolution of the initially acquired *P. aeruginosa* clone in the six most severely affected patients of our cohort until fatal outcome because of respiratory insufficiency and in the six mildest courses until replacement by another *P. aeruginosa* clone [22]. By 2022, the five patients alive enjoy a close-to-normal lung function and a normal family and working life despite an almost 40-year airway colonization with *P. aeruginosa*.

The genetic adaptation of *P. aeruginosa* to the CF airways was driven in these 12 patients by the extent of bronchial inflammation and structural damage of the CF

**Fig. 1**




Phylogenetic trees of serial *P. aeruginosa* isolates from CF airways. Microevolution of *P. aeruginosa* during chronic infection of CF airways is demonstrated by phylogenetic trees of sequential isolates based on maximum likelihood analysis. Isolates from persisting clonal lineages were collected from six patients (I–VI) over periods of 10–16 years. Isolates within a course were differentiated by ongoing numbers increasing over time. Genome sequencing allowed detection of point mutations and generation of consensus sequences that were applied to genome alignments for all isolates of a patient's course. From these alignments, rooted maximum likelihood trees were generated by applying 1000 bootstrap replicates and different nucleotide substitution models. The respective tree, which scored the highest overall maximum likelihood value, was selected for displaying the microevolution within an isolate course. Branch lengths represent the number of nucleotide substitutions per site that had occurred in the genome sequences of the corresponding isolates. To generate rooted phylogenetic trees, the sequence of the earliest isolate in a course was defined as the outgroup sequence from which the later isolates have evolved. The displayed trees demonstrate different microevolution modes with diversification mainly occurring in either one branch only or in several branches in parallel and with different substitution rates. Please note that maximum likelihood-based phylogenies do not display the time points of sampling of the isolates so that the time of persistence of branches or sublines cannot be seen as ordinate values in the figures. The increasing numbering of isolates according to the sampling date, however, allows the deduction of information on differentially evolved sublines with individual substitution rates that have persisted in the airways of the respective CF patient.

lungs at the time when *P. aeruginosa* took residence in the patient's airways [22]. The acquisition of loss-of-function mutations was prominent during fatal downhill courses of infection, whereas the genomic gain of metabolic versatility by horizontal gene transfer was typical for mild courses of infection [22]. Insertions of phages, transposons, integrons, and IS elements confer metabolic genes, virulence, and resistance determinants but also inactivate genes at the site of integration. Plasmids and more often mobile integrative and conjugative

**Table 1**

**Whole-genome sequencing of serial *P. aeruginosa* isolates from the airways of people with CF[a].**

| No. of patients | No. of sequenced isolates | No. of clone types | Time span of within-host evolution (years) | Reference |
|---|---|---|---|---|
| 2 | 45, 63 | 2 | 20, 23 | [17] |
| 34 | 474 | 36 | 1–8 | [21] |
| 4 | 26 | 6 | 17–19 | [53] |
| 12 | 262 | 12 | 10–35 | [22] |
| 1 | 40 | 1 | 8 | [54] |
| 39 | 443 | 52 | 0.2–10 | [23] |
| Hypermutator lineages | | | | |
| 2 | 13, 14 | 2 | 6, 20 | [20] |
| Transmissible lineages causing nosocomial spread at a CF center | | | | |
| 21 | 55 | 1 | 36 | [18,19] |
| 6 | 63 | 1 | 3–4 | [55] |
| Longitudinal isolates from the sinuses | | | | |
| 6 | 67 | 6 | 2–3 | [43] |

[a] Only studies were considered that sequenced the genomes of at least three serial isolates.

elements [32] modify virulence and fitness already by episomal copy number variation [33]. Particularly inducible genomic prophages were shown to regulate fitness, pathogenicity, and bacterial population density [34–36]. The gain and loss of genomic islands called RGPs, regions of genomic plasticity, were often observed in serial isolates from CF patients [33,37,38]. We still do not know much about the emergence of more complex structural variants in the *P. aeruginosa* chromosome, but according to own analyses of clone C and clone PA14 strain collections by physical mapping and long-read sequencing, large genome rearrangements occur frequently in CF isolates. For example, intramolecular transposition of an active IS element will typically lead to large chromosomal inversions accompanied by mutation, deletion, and/or duplication of sequence close to the breakpoints [39]. A further virtually still unexplored area is the microevolution of the epigenome of *P. aeruginosa*. SMRT sequencing of serial CF isolates did not only identify the methylated recognition sites of restriction-modification systems but also spatiotemporal genomic variation of the methylation rate (Fischer, unpublished).

## Microevolution of *P. aeruginosa* phenotype in cystic fibrosis airways

*P. aeruginosa* continuously diversifies its phenotype in CF lungs (Figure 2). During the phase of chronic colonization, the colonies of primary cultures of respiratory secretions will vary in morphotype, metabolic competence, QS signaling, virulence, motility, antimicrobial susceptibility, biofilm formation, and binding to human mucins and epithelia [40–44]. Most diversifications revert to the common phenotypes of an environmental isolate by repeated subculturing in vitro, but a few phenotypic traits are irreversibly fixed [44]. First, during the first years within the CF lung, a *P. aeruginosa* clone will reduce its repertoire of phage receptors and pyocins, indicating that the persistent clone succeeded to eliminate clonal competitors and to become resistant to phage attacks. In line with these observations, long-term cocolonization with two or more clones is rare. Second, the clone diversifies into planktonic and sessile subclones, the latter residing in the bronchial lumen embedded into own exopolysaccharides and human mucins. The sessile subclones lose flagella and pilins and become nonmotile and lowly virulent, whereas the planktonic cells retain their virulence effectors. With respect to the highly immunogenic LPS, strains modify or lose the O-antigen and modify their lipid-A promoting immune evasion, inflammation, and resistance to cationic antimicrobial peptides. Morphotypes reside side-by-side, the hallmarks being the more virulent and more drug-resistant small colony variants and the biofilm-forming mucoid colonies. The alginate-overexpressing phenotype is characteristic for CF isolates and very uncommon in other habitats. Mucoidy typically emerges during the first years of airway colonization, but five to fifteen years later, nonmucoid revertants appear that are often auxotrophic and attenuated in virulence. These slow-growing auxotrophic variants are seen in elderly CF patients with poor lung function. Being deficient in one or more metabolic pathways of amino acid biosynthesis, the auxotrophs thrive in the bronchial lumen of a heavily remodeled lung that provides plenty of amino acids as nutrients.

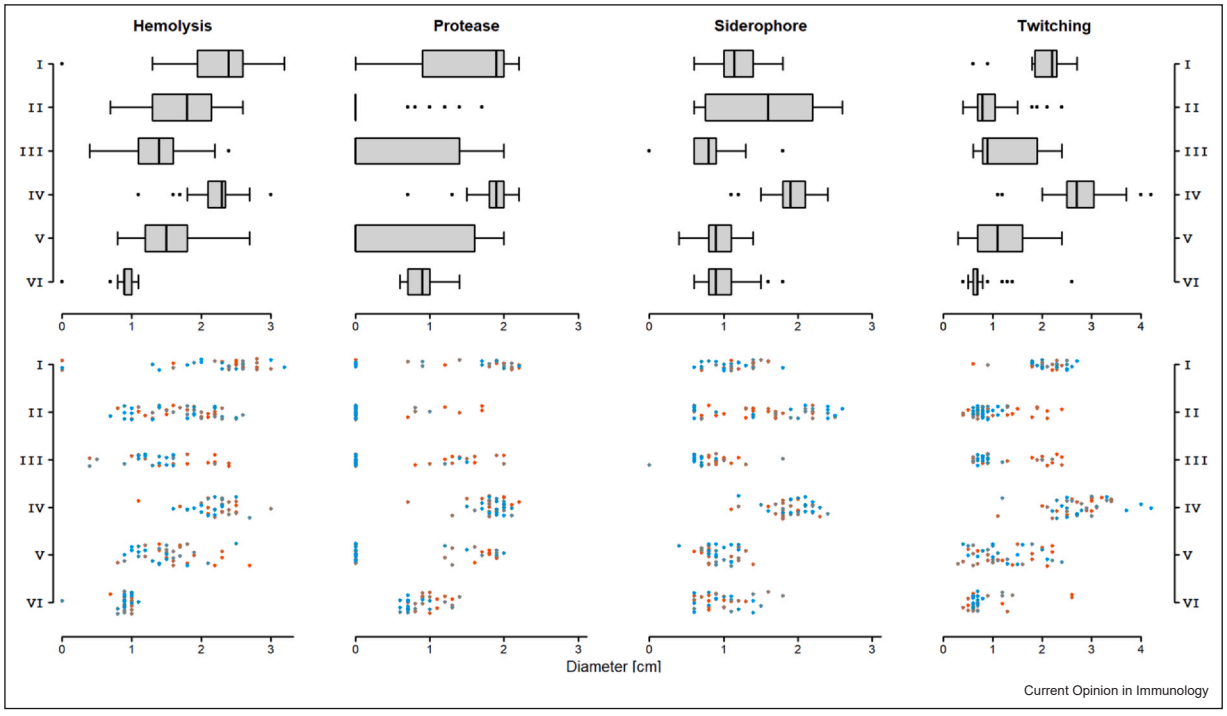
## Impact of microevolution on fitness

To examine the change of fitness during chronic colonization of CF airways, we simultaneously exposed clonal serial CF isolates to a range of habitats, that is,

- nutrient-poor and nutrient-rich liquid media representative for the typical aquatic habitat of *P. aeruginosa* [45],
- the lung model of murine and human-precision-cut lung slices (PCLS) [46],
- and human granulocytes, man's major cellular defense against infections with *P. aeruginosa* [47].

Serial isolates were selected from the six mildest and six most severe courses of chronic infection seen at the CF clinic, Hannover (see above). Growth and persistence of bacteria were monitored by amplicon sequencing of strain-specific single-nucleotide variants. Unexpectedly, the outcome of the competitive fitness experiments was similar for all investigated habitats. The serial CF isolates from the mild courses showed individual clone-specific patterns of growth and survival, although the early isolates were on average fitter than the midterm or

**Fig. 2**




Phenotypic characterization of sequential *P. aeruginosa* isolates from chronic CF lung infections. In order to investigate the microevolution of *P. aeruginosa* in the lungs of CF patients, extensive phenotyping of serial isolates was carried out in addition to genotyping. For presentation of the results of the phenotypic characterization, six long-term courses were selected as examples (I–VI) and the results of four different phenotypic assays (hemolysis, twitching, protease, and siderophore secretion) are displayed. For the evaluation of the various assays, the diameter of the grown colony was measured, the mean was calculated and normalized to the isolate with the largest diameter within the individual long-term course. All assays were performed in triplicate. The box plots in the upper panel demonstrate the high variability of secretion and motility phenotypes observed for the different courses. The lower panel shows the results for all single isolates. The isolates are displayed by varying colors according to the date of isolation (color gradient from orange for early isolates to blue for late isolates). Contrary to the literature, the plots demonstrate that serial *P. aeruginosa* progeny was not continuously losing all of their virulence phenotypes during long-term adaptation to the CF lung. Instead, virulent variants were often detected over the entire time course of the chronic infection.

late isolates to persist in the presence of their clonal relatives. In contrast, the strain collections from the severe courses showed a uniform outcome. The early isolates that differed least in their genomic profile from the environmental ancestor outcompeted their clonal progeny: the early isolates were growing better in nutrient-rich and nutrient-poor media, they outcompeted the progeny in murine as well as in human PCLS, and they were more resistant to intracellular killing by neutrophils. Hence, the outcome of the fitness experiments differed by the status of CF lung disease at the time when *P. aeruginosa* conquered the CF airways. In case of the mild courses, lung function and host defense were close to normal at onset of colonization. In case of the severe courses, lung function was already compromised when *P. aeruginosa* was acquired. The vicious cycle of infection, inflammation, and remodeling was already operating from the beginning, and under these living conditions, bacterial progeny with lower general fitness emerged that did not persist as well in an aquatic habitat or healthy lungs as its ancestor. In summary, the disease status of the CF lung habitat governed the adaptation of *P. aeruginosa* more strongly than the underlying clone type and its genetic repertoire.

## Comparison of within-host evolution in *M. tuberculosis*, *H. pylori*, and *P. aeruginosa*

The evolutionary dynamics within human hosts is different among the three taxa. Within susceptible human hosts, the molecular clock rates of nonmutator strains range between $10^{-8}$ and $5x10^{-7}$ nucleotide changes per site per year in *M. tuberculosis* [48] and range between $10^{-6}$ and $10^{-5}$ in both *H. pylori* [49] and *P. aeruginosa* [22]. Of the three species, *M. tuberculosis* shows the lowest rate of genome diversification during host–pathogen coevolution. There are currently 53 sublineages within the nine recognized major lineages of the clonal *M. tuberculosis* populations [50]. *H. pylori* and *P. aeruginosa* show a considerably higher genomic diversity within the individual human host and the global human population.

The naturally competent *H. pylori*, which grows in the human stomach, gains a substantial amount of sequence diversity by the uptake of short DNA fragments via recombination (see the article by Sebastian Suerbaum and his colleagues in this issue). In contrast, *P. aeruginosa* changes its genome organization by chromosomal uptake and release of mobile genetic elements [38], duplications, deletions, and large-scale inversions or transpositions [51,52]. Within CF hosts, the mutation rate of *P. aeruginosa* was highly variable ranging from five to eighty single-nucleotide substitutions per year in the core genome and in total two to sixteen major rearrangements of the accessory genome [22].

In summary, the within-host evolution triggers conversions of genotype and phenotype that attenuate the global fitness of the ubiquitous *P. aeruginosa* to thrive in any aquatic animate and inanimate habitat. However, during adaptation to the CF lung, *P. aeruginosa* retains enough flexibility to recognize its environment of host cells and polymers and to respond to selective pressures of host defense, antipseudomonal chemotherapy, and other microbes in the lung holobiome.

## CRediT authorship contribution statement

**Nina Cramer; Jens Klockgether**: Visualization, Writing – review & editing. **Burkhard Tümmler**: Writing – original draft, Writing – review & editing.

## Data Availability

Data will be made available on request.

## Conflict of interest

The authors declare no competing interests.

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