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Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance

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Abstract

Hypermutable or mutator microorganisms are those that have an increased spontaneous mutation rate as a result of defects in DNA repair or error avoidance systems. Over the last two decades, several studies have provided strong evidence for a relevant role of mutators in the evolution of natural bacterial populations, particularly in the field of infectious diseases. Among them, chronic respiratory infection with *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients was the first natural environment to reveal the high prevalence and important role of mutators. A remarkable positive selection of mutators during the course of the chronic infection has been reported, mainly as a result of the emergence of DNA mismatch repair system (*mutS*, *mutL* or *mutU*)-deficient mutants, although strains defective in the GO system (*mutM*, *mutY* and *mutT*) have also been observed. High frequencies of mutators have also been noted among other pathogens in the CF setting, particularly *Staphylococcus aureus* and *Haemophilus influenzae*. Enhanced antimicrobial resistance development is the most thoroughly studied consequence of mutators in CF and other chronic infections, although recent studies show that mutators may additionally have important effects on the evolution of virulence, genetic adaptation to the airways of CF patients, persistence of colonization, transmissibility, and perhaps lung function decline. Further prospective clinical studies are nevertheless still needed for an in-depth evaluation of the impact of mutators on disease progression and outcome.

Keywords: Adaptation, antibiotic resistance, chronic infection, cystic fibrosis, hypermutation, mutator, *Pseudomonas aeruginosa*, review

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Introduction

Hypermutable (or mutator) microorganisms are those that have an increased spontaneous mutation rate as a result of defects in DNA repair or error avoidance systems. Over the last few decades, several theoretical experiments, performed both *in vitro* and *in vivo*, have shown that mutator phenotypes confer an evolutionary advantage during bacterial adaptation to new environments or stressful conditions [1–5]. These investigations demonstrated that mutator cells, present in regular bacterial populations at a rate of the order of 10^{-5} as a consequence of spontaneous mutations in DNA repair genes, can be dramatically amplified by co-selection (hitchhiking) with adaptive mutations, playing a major role in bacterial evolution. Adaptation to the host immune system [6], antibiotic treatment [7] or viral parasites [8] has indeed been shown to be accelerated in mutator backgrounds in various experimental models. Further studies have provided strong evidence for a relevant role of mutators in the evolution of natural bacterial populations, particularly in the field of infectious diseases [9–12]. Among them, chronic respiratory infection (CRI) with *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients was the first natural model to reveal the high prevalence and important role of mutators [10]. Antimicrobial resistance development and bacterial adaptation during chronic infections are among the most relevant evolutionary traits linked so far to hypermutation in nature [10,13]. Current knowledge concerning the prevalence, mechanisms and consequences of bacterial mutators in CF CRI are reviewed.

Genetic Basis of Hypermutation

The mutator phenotype is consequence of a defect in one of the several DNA repair or error avoidance systems and, for

TABLE 1. Principal mutator genes, most functionally characterized in *Escherichia coli*

Gene	Product activity	Mutations produced	Mutator effect
mutD (dnaQ)	ϵ subunit of DNA pol III, proofreading activity	All base substitutions, frameshifts	Very strong
MMR system			
mutS	DNA mismatch recognition, binds mismatches	GC → AT, AT → GC, frameshifts	Strong
mutL	Interacts with MutS and MutH		
mutH	Endonuclease, nicks hemi-methylated GATC sequences		
uvrD	DNA Helicase II, strand displacement		
dam	DNA adenine methyltransferase, strand recognition		
GO system			
mutT	Nucleoside triphosphatase, prevents incorporation of 8-oxoG to DNA	AT → CG	Strong
mutM	DNA glicosylase, removes 8-oxoG from 8-oxoG-C mispairs	G:C → T:A	Weak
mutY	DNA glicosylase, removes A from 8-oxoG-A or A-G mispairs	G:C → T:A	Moderate
mutA	GlyV, glycyl tRNA	AT → TA, GC → TA, AT → CG	Weak–moderate
mutC	GlyW, glycyl tRNA	AT → TA, GC → TA, AT → CG	Weak–moderate
ung	Uracil glicosylase, removes U from U-G mispair	GC → AT	Weak–moderate
sodA, sodB	Superoxide dismutase, removes superoxide radicals	AT → TA	Weak
oxyR	Regulates hydrogen peroxide inducible genes	AT → TA	Weak
polA	DNA polymerase I	Frameshifts, deletions	Weak–moderate

this reason, the genes involved are generally referred to as (anti)mutator genes [14,15] (Table 1). Among them, the mismatch repair (MMR) system is especially important not only because its alterations are the most frequent cause of hypermutation in natural bacterial populations, but also because they are a frequent driver of human cancer [16].

The MMR system detects and repairs replication errors including any kind of mispairs and short insertions or deletions. The inactivation of any of the key genes involved (*mutS*, *mutL* and *mutH*, as well as *uvrD* or *mutU*) increases the rate of mutation from 100- to 1000-fold and the spectra of mutations produced include G:C → A:T and A:T → G:C transversions and deletions or insertions of 1–4 bp [17]. In *Escherichia coli*, the recognition of the mismatch by the MMR system relies on the methylation of the parental strand but not of the daughter strand at GATC sequences by Dam methyltransferase. For this reason, this system is also called the Dam-directed mismatch repair system. MutS recognizes and binds to the mismatch, MutL interacts with MutS and together activates the endonuclease MutH that cleaves the non-methylated strand at the GATC sequence. Unwinding by helicase II (UvrD) and excision and repair can then proceed in either the 5′ or 3′ direction. Not all microorganisms, including *P. aeruginosa*, have *dam* and *mutH* homologues, and therefore the recognition of the daughter strand is not based on DNA methylation but on alternative recognition pathways.

In addition to the activity of the MMR system preventing the accumulation of mutations as a result of replication errors, this system is also the most potent inhibitor of recombination between weakly and moderately diverged (homeologous) sequences, including genomes of related bacteria such as *E. coli* and *Salmonella* [18]. Therefore, the inactivation of the MMR system, in addition to increasing the mutation rates, increases the rates of homeologous recombination, facilitating the acquisition of exogenous DNA through horizontal gene transfer [19].

Mutations in *dnaQ* (*mutD*) coding for the epsilon subunit of the DNA polymerase III that has proofreading activity lead to a very strong mutator phenotype, increasing the mutation rate by up to 10 000-fold. The spectra of mutations produced include all types of base substitutions and frameshifts. Mutations in this gene also reduce the growth rate and have not been found in natural bacterial populations. Other mutator genes include *mutM*, *mutY* and *mutT*, which are involved in the system that prevents mutations caused by the oxidative lesion 8-oxodG (GO system). Mutations in *mutM* and *mutY* lead to a weak and moderate mutator phenotype, respectively, specifically increasing the rate of G:C → T:A transversions. On the other hand, mutations in *mutT* lead to a strong mutator phenotype increasing the rate of A:T → C:G transversions. Finally, other mutator genes comprise those involved in the prevention of oxidative damage produced by reactive oxygen species, such as *oxyR* and *sodA*, mutator tRNAs (*mutA* and *mutC*) [14] or *radA* and *pfpI*, as recently characterized in *P. aeruginosa* [20,21].

In addition to the stable mutator phenotypes produced by the alteration of the mutator genes, under particular circumstances (e.g. when DNA is damaged), a transient mutator phenotype is produced by the induction of the error-prone DNA polymerases (DNA polymerases IV and V) as part of the SOS response [22,23]. Some antibiotics may actually induce a transient mutator phenotype through this mechanism, promoting the development of antimicrobial resistance [24–26].

Laboratory and Theoretical Evidence for a Role of Hypermutation in Adaptive Evolution

Laboratory and theoretical approaches have shown that, under particular circumstances, such as exposure to new environments or stressful conditions, mutator cells maybe

selected in a bacterial population by hitchhiking with adapting mutations [1–3,27]. For example, Sniegowski *et al.* [2] found that, in *E. coli* populations evolving for 10 000 generations in a glucose-limited environment, there is a frequent ascent to dominance of mutator variants. These findings were consistent with simultaneously reported computer simulations of bacterial evolution [3]. A good example of how mutator cells can be amplified in a bacterial population by hitchhiking with adapting mutations was provided by Mao *et al.* [4]. In that study, it was found that when *E. coli* populations are subjected to a one-step mutation selection process (i.e. culture in a medium in which only particular mutant colonies can grow), hypermutable variants were amplified in the population from approximately 0.001% to 0.5% and, when they were subjected to two consecutive steps of mutant selection, the amplification reached 25–100%. A similar amplification of MMR-deficient mutator cells has been found after selection for recombinants in interspecies mating between *Salmonella* and *E. coli* [19]. These results demonstrate that horizontal gene transfer may also select for hypermutable variants during adaptive evolution as a result of the increased rate of homeologous recombination of MMR-deficient cells. Finally, using a murine model of *E. coli* intestinal colonization, Giraud *et al.* [5] found that hypermutation was initially beneficial because it allowed a faster adaptation to the mouse gut environment, although this advantage disappeared once adaptation was reached, and the transmissibility of the hypermutable strains was then considerably reduced. More recently, Pal *et al.* [8] showed that the co-evolution with viral parasites could be an important driver of the evolution of bacterial mutation rates in laboratory populations of *Pseudomonas fluorescens*. After fewer than 200 bacterial generations, 25% of the populations co-evolving with phages had evolved ten- to 100-fold increases in mutation rates owing to mutations in MMR genes; no populations evolving in the absence of phages showed any significant change in mutation rates. Furthermore, mutator populations had a higher probability of driving their phage populations to extinction, strongly suggesting that mutators have an advantage against phages in the co-evolutionary arms race.

Further laboratory experiments, using *P. aeruginosa* as model microorganisms, have shown that hypermutation has major effects on the evolution of bacterial cooperation and virulence [28,29].

Prevalence of Mutators in Natural Bacterial Populations

The prevalence of mutators in natural populations was first explored in *E. coli* and *Salmonella*, and was found to be higher than expected (approximately 1%) [9,30]. These findings, in concordance with the results obtained via *in vitro* experiments, suggested that hypermutation could act as a mechanism for acceleration of bacterial evolution in nature. The first evidence for a specific environment driving the selection of hypermutable strains in natural populations was obtained in a study of *P. aeruginosa* CRI in CF patients in which the prevalence of hypermutable strains was by far the highest ever found in nature [10]. Further work revealed that *Staphylococcus aureus* and *Haemophilus influenzae* from CF patients are also frequently hypermutable [31–33]. The association between mutators and chronic infections extends beyond CF because hypermutable *P. aeruginosa* has also been found to be very prevalent in other CRI, such as those occurring in patients with bronchiectasis or chronic obstructive pulmonary disease (COPD) [34,35]. On the other hand, the prevalence of mutator *P. aeruginosa* has been shown to be low in acute infections [10,36]. Additional links to chronicity might also be seen in the case of *E. coli* because the inactivation of the MMR system was shown to increase the chronic persistence of urinary tract infections (UTI) in mouse models [37], and mutator strains appear to be particularly frequent among clinical UTI isolates [38]. Mutator strains have additionally been documented in natural populations of *Helicobacter pylori* [39], *Neisseria meningitidis* [40] *Streptococcus pneumoniae* [41], *Mycobacterium tuberculosis* [42], *Klebsiella pneumoniae* [43], *Vibrio parahaemolyticus* [44] and *Stenotrophomonas maltophilia* [45], with variable prevalences. Particularly noteworthy, a link between hypermutation and epidemicity was demonstrated for *N. meningitidis* [40,46]. The epidemicity of this microorganism is dependent upon its success in adapting to different human hosts, which is achieved by stochastically altering its surface composition by a mechanism of phase variation mediated by mutations in homopolymeric DNA direct repeats. Remarkably, approximately 50% of the highly epidemic and virulent serogroup A *N. meningitidis* isolates were found to be defective in the MMR system, expressing a rate of phase variation >100-fold higher than wild-type isolates. A possible link between hypermutation and the success of the epidemic, highly disseminated W-Beijing clone of *M. tuberculosis* has also been studied. Ebrahimi-Rad *et al.* [42] found that W-Beijing genotype strains displayed unique missense alterations in three putative mutator genes, including two homologues of *mutT*, although other studies reported no differences in the mutation rates of Beijing and non-Beijing strains [47].

Mutators in CF CRI

CF, caused by mutations in the gene that encodes the CF transmembrane conductance regulator, is the most prevalent

autosomal-recessive hereditary disease in Caucasian populations. Amongst other repercussions, this defect leads to an alteration of respiratory secretions and determines a predisposition for chronic bronchopulmonary colonization/infection, which is the main driver of the high morbidity and early mortality of CF patients [48]. Colonization with *S. aureus* and *H. influenzae* is frequent in children aged less than 10 years, whereas *P. aeruginosa* is by far the most relevant pathogen in adults with CF and is responsible for the progressive bronchopulmonary deterioration [49].

In an initial study published in 2000, an extremely high prevalence (20% of isolates, 37% of patients) of *P. aeruginosa* mutator strains was found in chronically colonized CF patients from Spain [10]. These numbers notably contrasted with an absence of mutator strains in acute *P. aeruginosa* nosocomial infections from the same study, and with the existing data on *E. coli* and *Salmonella* natural populations, in which 1% of mutators was already considered to represent a high prevalence [9,30].

Further studies confirmed and extended these initial observations. In a cross-sectional study, Ciofu *et al.* [50] detected mutator strains in 54% of Danish CF patients who were chronically colonized with *P. aeruginosa*. The same study included also a longitudinal (up to 25 years) evaluation of the prevalence of *P. aeruginosa* mutator strains in CF patients; remarkably, the proportion of hypermutable isolates increased from 0% at onset/early colonization to 65% after 20 years of chronic colonization.

Other studies [13,51,52] had noted a certain prevalence (approximately 5–10%) of hypermutable isolates already at onset/early colonization. Indeed, the reported prevalence of mutators during initial colonization could well reflect their basal prevalence in the environment (i.e. the main source for *P. aeruginosa* colonizing CF patients) because 6% of isolates from environmental samples were found to be mutators in a recent study [51]. Nevertheless, an overwhelming positive selection of mutators during the course of CF chronic infection has always been documented [13,50,52]. Furthermore, the positive selection of mutators appears to be a common feature of chronic infections, and not exclusively of CF-associated infections, because an extremely high prevalence (53% of isolates, 57% of patients) of hypermutable strains has been documented in patients with COPD or bronchiectasis [34]. Taken together, the available data [10,36] apparently indicate a negative selection of mutators in acute nosocomial infections, with a prevalence of ≤1%.

Finally, Kenna *et al.* [51] investigated the prevalence of mutators in representative isolates from different epidemic CF strains. The proportion found (2/15; 13%) was not significantly higher than that documented for strains from onset/early colonization in the same study. Indeed, the transmission of mutator strains between CF patients has never been demonstrated so far.

Regarding the genetic basis of hypermutation in *P. aeruginosa* strains from CF patients, by far the most widely investigated mechanism is that of the MMR system. Up to 60–90% of the mutator isolates from CF patients have a defective MMR system, mainly caused by mutation of *mutS* or *mutL*, and less frequently of *uvrD* (*mutU*) [13,52–54]. Considerably less information is available on the potential involvement of other DNA repair systems in hypermutability. *P. aeruginosa* was shown to have a conserved GO system (*mutM*, *mutY* and *mutT*) [55], and a few mutator isolates from CF patients failed to yield a positive result for *mutY* amplification in an initial study [10]. More recently, Mandsberg *et al.* [56] and Ciofu *et al.* [54] have detected some *mutT* and a *mutY* isolates among mutator strains from CF patients.

A higher prevalence of hypermutable strains in the CF setting has also been noted for *S. aureus* and *H. influenzae*, demonstrating that this phenomenon is not restricted to *P. aeruginosa* only. Prunier *et al.* [31] found that approximately 14% of the *S. aureus* isolates from CF patients were hypermutable in contrast to the approximately 1% from non-CF patients. The same results (14% vs. 1%) were obtained by Román *et al.* [32] for *H. influenzae*. More recently, Besier *et al.* [57] noted that *S. aureus* mutator phenotypes were particularly frequent among thymidine-dependent small-colony variants which are known to play an important role in the pathogenesis and persistence of CF CRI. Regarding the genetic basis of hypermutation in these microorganisms, the most frequent cause also appears to be a defective MMR system due to *mutS* or *mutL* mutations [31,33,57]. Finally, a recent study has also documented the presence of *mutS*-deficient mutator strains of *S. maltophilia* in chronically colonized CF patients [45].

As discussed below, enhanced antimicrobial resistance development has been the most thoroughly studied consequence of mutators in CF and other chronic infections, although recent work shows that mutators may additionally have important effects on the evolution of virulence, genetic adaptation to the airways of CF patients, persistence of colonization, transmissibility, and perhaps lung function decline [10,13,50,52,58–63]. Table 2 summarizes the features associated with mutator strains from chronically infected CF patients.

Mutators and Antibiotic Resistance

Over last decade, hypermutation has been increasingly recognized as a relevant problem for antimicrobial therapy

TABLE 2. Features associated to mutator strains from chronically infected cystic fibrosis (CF) patients

Publication year	Species	Feature	Description	Reference
2000	*Pseudomonas aeruginosa*	Antibiotic resistance	Mutator strains showed higher resistance percentages to ticarcillin, ceftazidime, imipenem, gentamicin, tobramycin, norfloxacin, and fosfomycin	Oliver *et al.* [10]
2003	*Staphylococcus aureus*	Antibiotic resistance	Mutator strains were more frequently resistant to macrolides, due to ribosomal mutations	Prunier *et al.* [31]
2004	*Haemophilus influenzae*	Antibiotic resistance	Mutator strains were linked to mutation-mediated resistance, particularly ciprofloxacin resistance and the β-lactamase negative ampicillin resistance (βLNAR) phenotypes	Román *et al.* [32]
2005	*Streptococcus pneumoniae*	Antibiotic resistance	Significant association of mutators with antibiotic resistance was not observed	del Campo *et al.* [58]
2005	*Pseudomonas aeruginosa*	Antibiotic resistance	Mutator strains showed higher MIC_{50} and MIC_{90} of ceftazi dime, piperacillin-tazobactam, aztreonam, meropenem, tobra mycin, and ciprofloxacin	Ciofu *et al.* [50]
		DNA oxidation	Higher levels of 8-oxo-2′-deoxyguanosine	
2007	*Pseudomonas aeruginosa*	Antibiotic resistance	Mutator strains showed higher antibiotic resistance rates	Hogardt *et al.* [59]
		Virulence	Mutator strains showed reduced cytotoxicity	
		Fitness	Mutator strains showed reduced survival in tap water	
2007	*Pseudomonas aeruginosa*	Fitness	Mutator strains showed reduced fitness *in vitro* and in a murine model of chronic respiratory infection	Montanari *et al.* [52]
2007	*Pseudomonas aeruginosa*	Multidrug-resistance (MDR)	Almost all MDR strains (resistant to β-lactams, fluoroquinoles, and aminoglycosides) from CF patients were mutators	Henrichfreise *et al.* [60]
2007	*Pseudomonas aeruginosa*	Colistin susceptibility	Mutator strains showed lower colistin MICs than non-mutator strains	Macia *et al.* [61]
2008	*Pseudomonas aeruginosa*	Accumulation of adaptive mutations	Mutator strains acquired a higher number of mutations in 34 genes potentially involved in adaptation to the airways of CF patients	Mena *et al.* [13]
2008	*Pseudomonas aeruginosa*	Lung function	Patients colonized by mutator strains had poorer lung function (% FEV_1)	Waine *et al.* [62]
		Mucoid phenotype	Mutator strains showed more frequently the mucoid phenotype	
2009	*Pseudomonas aeruginosa*	Antibiotic resistance	Mutator strains showed higher resistance percentages to ticarcillin, ticarcillin+clavulanate, piperacillin, piperacillin-tazobactam, cefsulodin, aztreonam, ceftazidime, imipenem, tobramycin, amikacin, and ciprofloxacin	Ferroni *et al.* [63]
		Lung function	Mutator strains acquired additional resistance mechanisms faster than non-mutator strains Patients colonized by mutator strains had poorer lung function (% FEV_1)	

[64,65]. The first evidence for a role of mutators in antimicrobial resistance development was obtained from the model of *P. aeruginosa* CRI in CF patients [10]. One of the most striking characteristics of *P. aeruginosa* is its extraordinary ability to acquire antibiotic resistance through the selection of mutations in chromosomal genes [66]. This is an especially critical factor in the management of chronic infections such as those occurring in CF patients. After years of intensive antibiotic chemotherapy in an effort to control the negative outcome of chronic colonization, sequential development of resistance to most antibiotics occurs frequently. Not surprisingly, resistance rates among *P. aeruginosa* isolates from CF patients are substantially higher than those found in isolates from other settings, including those from patients in intensive care units [67]. Subsequent to their first description [10], mutators were shown to play a major role in the high resistance rates of CF isolates, thus representing an important negative factor for the treatment of the CF CRI. Furthermore, when the rates of antimicrobial resistance were analyzed, mutators were found to be much more frequently resistant than non-mutators to each of the eight antipseudomonal agents tested. Similar results were obtained later by Ciofu *et al.* [50] in a large collection of CF *P. aeruginosa* isolates from Denmark, and, more recently, by Ferroni *et al.* [63] in CF isolates from France. The latter study additionally analyzed the effect of hypermutation on the time required for antibiotic resistance to appear, and demonstrated that mutator strains acquired additional resistance mechanisms much more quickly than non-mutator strains. In a highly illustrative work, Henrichfreise *et al.* [60] investigated a collection of 22 multidrug resistant (MDR) strains (resistant to β-lactams, fluoroquinolones and aminoglycosides) from a multicentre study in Germany, 12 obtained from CF patients and ten from non-CF patients. Remarkably, almost all (11/12) of the CF strains were mutators, in which MDR had emerged by combination of multiple mutation-mediated resistance mechanisms. By contrast, MDR strains from non-CF patients were mostly non-mutators in which horizontally acquired resistance played a mayor role. Nevertheless, a strong link between *P. aeruginosa* hypermutation and antibiotic resistance has also been documented for non-CF CRI, such as those occurring in patients with bronchiectasis or COPD [34].

A strong correlation between hypermutation and antibiotic resistance has also been observed for *S. aureus* and

H. influenzae in the CF setting. Prunier *et al.* [31] noted that a high proportion (53%) of the *S. aureus* isolates from CF patients was resistant to erythromycin. Interestingly, more than half of the resistant isolates did not contain any acquired macrolide resistance gene but rather contained mutations in *rrl* (23S rRNA), *rplD* (L4 protein) or *rplV* (L22 protein), which is very infrequent in macrolide-resistant *S. aureus* isolates from other sources. This high prevalence of mutational macrolide resistance was found to be associated with a high prevalence of hypermutable strains in CF patients. Similarly, Román *et al.* [32] found a strong correlation between the high prevalence of hypermutable *H. influenzae* strains in CF patients and high rates of mutational antibiotic resistance. For example, CF isolates comprised significantly higher percentages of β-lactamase-negative ampicillin-resistant isolates than non-CF isolates, whereas the rates of β-lactamase-producing isolates was similar in both groups. Of particular concern was the observation that up to 20% of the *H. influenzae* strains from CF patients were resistant to ciprofloxacin. It is worth noting that ciprofloxacin resistance in *H. influenzae* is extremely infrequent in other settings and, indeed, was not found in any of 188 non-CF strains from the same study.

In addition to the clear statistical link between hypermutation and antibiotic resistance (i.e. mutators are more frequently antibiotic resistant) found after the analysis of collections of clinical isolates from CF patients, several *in vitro* and *in vivo* experiments, as recently reviewed [68], further highlight the dramatic consequences of hypermutation when occurring in microorganisms such as *P. aeruginosa* that are genetically equipped to acquire efficient resistance to most antibiotics by mutations in chromosomal genes [7,56,69–75].

Beyond Antibiotic Resistance: Role of *P. aeruginosa* Hypermutation in Virulence, Fitness, Transmissibility, Persistence and Genetic and Metabolic Adaptation During CF Lung Infection

The establishment of *P. aeruginosa* CRI is mediated by a complex adaptive process that includes physiological changes produced by the activation of specific regulatory pathways, including the induction of the biofilm mode of growth or the differential expression of virulence genes [76], as well as genetic changes leading to the selection of an important number of adaptive mutations required for long-term persistence [77–79].

Although patients suffering from CRI are generally infected with a single *P. aeruginosa* strain persisting in most cases all through the patient's life [80], one of the hallmarks of these infections is the emergence, and fixation over time, of multiple phenotypic variants from the underlying clonal populations [10], a process known as adaptive radiation [81]. Many of the selected phenotypes have been clearly linked to the adaptation to the lung environment, thereby favouring the lifelong persistence of CRI [79]. Long-term persistence in the airways of CF patients appears to be driven by the selection of multiple, frequently loss-of-function, mutations leading to an overall pattern of increased antimicrobial resistance, adapted virulence (reduced acute injury but enhanced chronic inflammation) and specific metabolic adaptation to growth under microaerobic conditions created by suppurative secretions in the lungs of CF patients [77,82–84].

The intense genetic adaptation process taking place during the establishment of CRI has been recently quantified [77]. Whole-genome sequencing of early (6 month-old) and late (96 month-old) *P. aeruginosa* isolates from a CF patient revealed the acquisition of up to 68 mutations during the establishment of the CRI. A clear signal of positive selection was demonstrated because an extraordinarily high ratio of nonsynonymous to synonymous mutations per site was documented. Indeed, many of the mutations detected lead to the loss of function of the affected genes, which, of note, were frequently involved in bacterial virulence. The work was completed by the analysis of a collection of sequential *P. aeruginosa* isolates from 29 additional CF patients; 24 of the genes that had been mutated early in patient 1, and ten additional genes that had been shown to be candidates for mutation in many CF infections, were sequenced. Again, the signal of positive selection of mutations in these genes was overwhelming because only five synonymous mutations accompanied 103 nonsynonymous mutations.

Using the same collection of strains, a recent study demonstrated that this intense genetic adaptation process is catalyzed by mutator strains [13]. Indeed, the presence of the adaptive traits was found not to be homogeneous among the CF isolates; by contrast, it was significantly concentrated in the MMR system-deficient lineages. The documented differences in the rates of accumulation of mutations per year of infection were certainly overwhelming: although sequential non-mutator lineages acquired a median of only 0.25 mutations in the 34 studied genes per year of infection, sequential mutator lineages accumulated over three mutations per year, representing a 13-fold higher rate. Consequently, the proportion of mutator isolates was dramatically increased among CF isolates containing a growing number of total mutations. For example, as illustrated in Fig. 1, the proportion of mutators increased from 16.7% when all isolates were considered to 87.5% in isolates containing at least seven mutations.

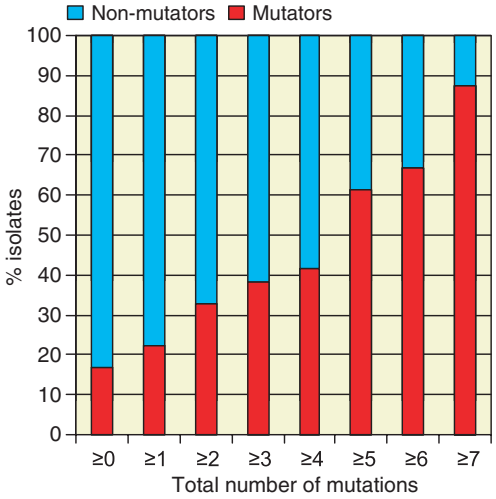

FIG. 1. Percentages of mutators and non-mutators among cystic fibrosis isolates showing a growing number of mutations in 34 genes, as described by Mena *et al.* [13].

Interestingly, the increased accumulation of mutations in mutator isolates was not the consequence of an over-representation of mutations in genes involved in antimicrobial resistance, which is the only adaptive trait that had been linked so far to hypermutation in isolates from CF patients, demonstrating that hypermutation has a generalized effect on *P. aeruginosa* evolution and adaptation during CRI. For example, of the two genes showing a higher number of mutations in late CF isolates, one (*mexZ*) was involved in antibiotic resistance and the other (*lasR*) was involved in quorum sensing regulation and virulence; the acquisition of mutations in both genes was equally greatly enhanced in mutators compared to non-mutators. Further recent transcriptome and proteome analysis of sequential isolates from CF patients has revealed that mutators play also a role in the metabolic adaptation of *P. aeruginosa* during CRI, which includes up-regulation of the anaerobic arginine deaminase pathway, anaerobic and microaerobic respiration, and the tricarboxylic acid cycle and glyoxylate shunt [82]. Fig. 2 shows a schematic representation of the evolution (adaptation) of *P. aeruginosa* during CF chronic lung infection and the role of mutators as catalyzers of the process.

These results are consistent with other recent findings in the CF mouse model of chronic colonization, in which *P. aeruginosa* mutator strains were shown to favour long-term persistence even in the absence of antimicrobial therapy [85]. Indeed, as illustrated in Fig. 3, the *mutS*-deficient strain clearly outcompeted wild-type PAO1 after long-term colonization, most likely through the increased rate of acquisition of adaptive mutations. These data are also in

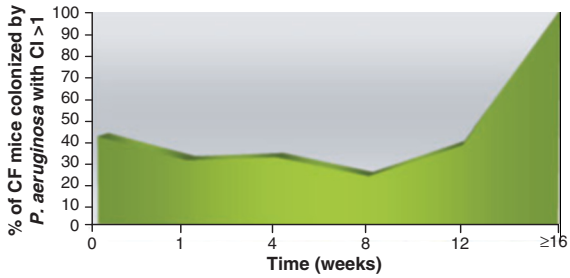

FIG. 3. Percentages of cystic fibrosis (CF) mice showing *Pseudomonas aeruginosa*-positive cultures in which PAO*ΔmutS* outcompetes PAO1 (Competition Index, CI > 1) over time, as described by Mena *et al.* [85].

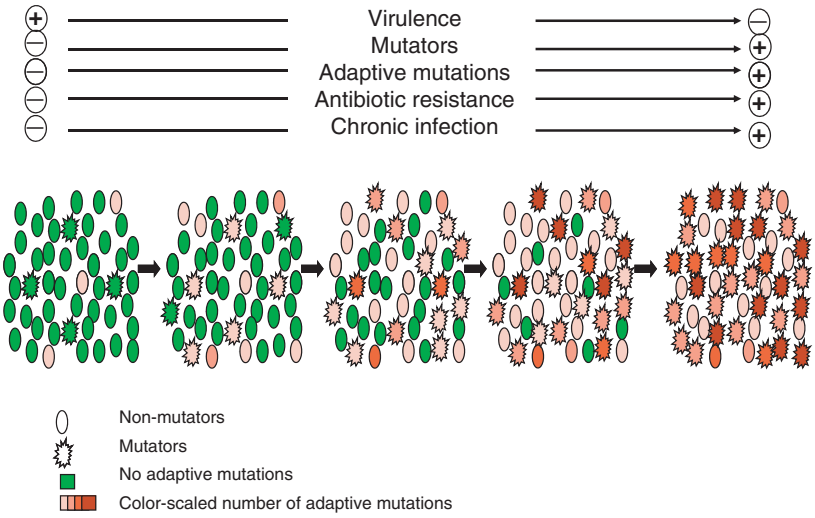

FIG. 2. Schematic representation of the evolution (adaptation) of *Pseudomonas aeruginosa* during cystic fibrosis chronic lung infection and the role of mutators as catalyzers of the process.

agreement with recent studies demonstrating that the inactivation of the MMR system favours the emergence of multiple *P. aeruginosa* phenotypic variants *in vitro*, including *lasR* and *mucA* (*mucA22*) mutants [86–88], the typical markers of CF lung infection.

It should be noted, however, that the shortcut to genetic adaptation to the airways of CF patients mediated by mutators is not expected to be free of cost. Although the acquisition of mutations under a positive selective pressure should speed up adaptation, it may also lead to the accumulation of mutations deleterious for secondary environments. Indeed, adapted mutator lineages recovered from mice with CF were previously shown to reduce transmissibility [85]. Similarly, cross-transmission of mutator lineages among CF patients has not yet been observed, in contrast to documented dissemination of non-mutator strains with early adaptive mutations such as those in *lasR* or *mucA* [54]. Moreover, mutator isolates recovered from late CRI in CF patients have been shown to have a reduced fitness and virulence once they are retrieved from their primary lung environment [52,59]. Finally, recent studies have evaluated the effect of hypermutation in the development and evolution of *P. aeruginosa* biofilms, which are a hallmark of chronic infections. Spontaneous mutation has indeed been shown to be increased during biofilm growth of wild-type strains as a consequence of endogenous oxidative stress, and the generated phenotypic diversity is considered to play a major role in the persistence of biofilm-driven infections [89–91]. A recent study by Conibear *et al.* [92] has shown that MMR-deficient strains, accumulating further spontaneous mutations in biofilms, notably promote the growth and evolution of the characteristic microcolonies.

Clinical Impact of Mutator Strains of *P. aeruginosa* in CF

As discussed above, the tight link between mutator phenotypes and antimicrobial resistance in itself should be considered as having major negative clinical consequences, severely compromising the efficacy of currently used antipseudomonal agents. Perhaps less obviously, the clear link to several other adaptive mutations assumed to play a role in the persistence of the chronic infection might also be considered as mutator-related negative clinical outcomes.

Nevertheless, the quantification of the effect of mutators on disease progression and deterioration of the lung function is certainly not an easy task; it was attempted in two recent studies carried out by Waine *et al.* [62] and Ferroni *et al.* [63]. Waine *et al.* [62] investigated 40 CF patients from the UK and found a statistically significant poorer lung function in CF patients in whom mutator strains where detected (40%), showing a lower % predicted FEV1 (40 vs. 57) and a lower mean FEV1/FVC (53 vs. 64).

Similarly, Ferroni *et al.* [63] investigated 36 CF patients (with mutators detected in 50%) from France, also documenting a significant association with poorer lung function (median % FEV_1 of 43 vs. 69). Although the presence of mutators is thus clearly linked to a poorer lung function, as observed in both studies, several factors complicate the assessment of mutators as independent risk factors. Among them, the duration of the CRI appears to be particularly relevant; it is well known that both the prevalence of mutators and the deterioration of the lung function progressively increase with the duration of the CRI, making it difficult, without further longitudinal clinical studies, to determine whether mutators are the cause or their presence is the consequence of lung function decline.

The presence of mixed mutator–non-mutator populations is also a potentially relevant factor; the probability that a given CF patient is catalogued as 'colonized with mutators' should thus be highly influenced by the number of sputum samples examined, and, as noted by Waine *et al.* [62], patients with poorer lung function contributed many more samples to the study.

Future Directions

The consequences of the high prevalence of mutators have been mostly studied with respect to *P. aeruginosa*, whereas corresponding information regarding other CF pathogens is still limited. Certainly, one of the most important aspects to be considered in future studies is an investigation of the optimal therapeutical approaches that should be used in attempts to minimize the impact of mutators.

Avoiding the selection of mutator strains in the first place (i.e. before adaptation to chronicity is reached) should be the ideal approach. Whether the early aggressive antibiotic treatments, as currently recommended for lung infections with *P. aeruginosa* in CF patients, would also be beneficial for this purpose needs to be further explored. Finally, additional studies are required to determine the best therapeutic strategies for avoiding the development of multiple antimicrobial resistance once mutator strains have emerged in the lungs of CF patients. The acquisition of adaptive mutations and lung function decline are also among the consequences attributed to *P. aeruginosa* mutators in chronic infections in CF patients, although further prospective clinical studies are still needed for a thorough evaluation of the impact of mutators on

disease progression. These studies should include a larger longitudinal and cross-sectional evaluation of the dynamics of mutator populations in the CF lung. Moreover, to fully understand the potential role of mutators in the physiopathology of CF disease, we still need to gain insight into the spatial and physical distribution of mutators within the lungs. The determination of whether they are mainly selected in the conductive or respiratory zones, and from planktonic populations or alginate-producing biofilm communitites, is expected to be decisive with respect to their contribution to the lung disease [93]. An additional evaluation of the inter-host dynamics of mutator in comparison with non-mutator populations should be useful for assessing the impact of hypermutation on the epidemiology and control of chronic infections in CF centres.

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Transparancy Declaration

The authors declare that there are no conflict of interests.

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