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Genetic adaptation of *Pseudomonas aeruginosa* during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogeneous genetic backgrounds emerge in *mucA* and/or *lasR* mutants

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During the chronic lung infection of patients with cystic fibrosis (CF), *Pseudomonas aeruginosa* can survive for long periods due to adaptive evolution mediated by genetic variation. Hypermutability is considered to play an important role in this adaptive evolution and it has been demonstrated that mutator populations are amplified in the CF lung by hitchhiking with adaptive mutations. Two of the genes that are frequently mutated in isolates from chronic infection are *mucA* and *lasR*. Loss-of-function mutations in these genes determine the phenotypic switch to mucoidy and loss of quorum sensing, which are considered hallmarks of chronic virulence. The aims of our study were to investigate (1) the genetic background of the *P. aeruginosa* subpopulations with non-mutator, weak or strong mutator phenotype and their dynamics during the chronic lung infection, and (2) the time sequence in which the hypermutable, mucoid and quorum-sensing-negative phenotypes emerge during chronic lung infection. For these purposes the sequences of *mutS*, *mutL*, *uvrD*, *mutT*, *mutY* and *mutM* anti-mutator genes as well as of *mucA* and *lasR* were analysed in 70 sequential *P. aeruginosa* isolates obtained from the respiratory secretions of 10 CF patients (one to three isolates per time point). Analysis of the genetic background of the mutator phenotype showed that *mutS* was the most commonly affected gene followed by *mutL* in isolates with strong mutator phenotype. The *mutT*, *mutY*, *mutM* genes were affected in isolates with low fold-changes in the mutation frequencies compared to the reference strain PAO1. Isolates with non-mutator, weak or strong mutator phenotype were represented at all time points showing co-existence of these subpopulations, which suggests parallel evolution of the various mutators in the different focal niches of infection in the CF lung. Mutations in *mucA* and *lasR* occurred earlier than mutations in the anti-mutator genes, showing that hypermutability is not a prerequisite for the acquisition of mucoidy and loss of quorum sensing, considered hallmarks of chronic virulence. Significantly higher mutation rates and MICs of ceftazidime, meropenem and ciprofloxacin were found for isolates collected late (more than 10 years) during the chronic lung infection compared to isolates collected earlier, which suggests an amplification of the mutator subpopulation by hitchhiking with development of antibiotic resistance. Similar evolutionary pathways concordant with adaptive radiation were observed in different clonal lineages of *P. aeruginosa* from CF patients.

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Abbreviations: CF, cystic fibrosis; GO, 7,8-dihydro-8-oxo-deoxyguanosine; Hp, strong mutator phenotype; MMR, methyl-directed mismatch-repair; nhp, non-mutator phenotype; wHp, weak mutator phenotype; PMN, polymorphonuclear leukocyte.

Two supplementary tables are available with the online version of this paper.

INTRODUCTION

During the chronic lung infection of patients with cystic fibrosis (CF), *Pseudomonas aeruginosa* can survive for long periods under the challenging selective pressure imposed by the immune system and the antibiotic treatment, due to

a biofilm mode of growth (Høiby *et al.*, 2008) and adaptive evolution mediated by genetic variation (Smith *et al.*, 2006). It has been shown that hypermutability plays an important role in this adaptive evolution and it has been demonstrated that mutator populations are amplified by hitchhiking with adaptive mutations (Mena *et al.*, 2008). When *P. aeruginosa* strains have survived in the CF lung for more than 30 years, they may have experienced 65 000 divisions, during which time niche specialists have evolved that are well adapted to survive in CF lungs, but have less capability to survive elsewhere. Accumulation of mutations leads to phenotypic changes in CF isolates such as increased alginate production and occurrence of mucoid variants (Govan & Nelson, 1993), loss of quorum sensing (Smith *et al.*, 2006; D'Argenio *et al.*, 2007), loss of motility (Mahenthiralingam *et al.*, 1994), loss of effector proteins of the type III secretion system (Jain *et al.*, 2004), loss of the O-antigen components of the lipopolysaccharide (Hancock *et al.*, 1983), reduced virulence (Luzar & Montie, 1985), reduced capacity for *in vitro* biofilm formation (Lee *et al.*, 2005) and increased antibiotic resistance (Ciofu *et al.*, 2001).

CF *P. aeruginosa* isolates with mutator phenotype exhibit a broad range of mutation frequencies from 1000 to a few fold-changes compared to the mutation frequencies of the laboratory strain PAO1 (Oliver *et al.*, 2000; Ciofu *et al.*, 2005; Hogardt *et al.*, 2007; Kenna *et al.*, 2007; Mena *et al.*, 2008). A strong mutator in clinical isolates has been defined as a strain with mutation frequencies at least 20-fold higher than strain PAO1 ($\geqslant 2\times10^{-7}$ for rifampicin). A strain with lower fold-changes can be considered a weak mutator ($<2\times10^{-7}$ and $\geqslant 2\times10^{-8}$), while strains with mutation frequencies similar to or lower than PAO1 are considered non-mutators ($<2\times10^{-8}$).

P. aeruginosa CF isolates with strong mutator phenotype have been repeatedly shown to be caused by loss-of-function mutations in genes related to DNA repair belonging to the methyl-directed mismatch-repair system (MMR system), which includes *mutS*, *mutL* and *uvrD* (Oliver *et al.*, 2002; Montanari *et al.*, 2007; Hogardt *et al.*, 2007; Mena *et al.*, 2008). There have been indications that genes (*mutT*, *mutY* and *mutM*) belonging to the system that prevents or repairs the mutations produced by the oxidative lesion mediated by 7,8-dihydro-8-oxo-deoxyguanosine (8-oxo-dG or GO) are also involved in the occurrence of mutators (Oliver *et al.*, 2000; Hogardt *et al.*, 2007). We have recently reported CF *P. aeruginosa* isolates with mutator or weak mutator phenotype due to inactivation of *mutT* and *mutY* genes (Mandsberg *et al.*, 2009). Novel mutator genes PA0140 (*ahpF*), PA4609 (*radA*), PA3002 (*mfd*), PA3003 and PA0750 (*ung*) have been recently reported to play a role in hypermutability, especially in isolates with weak mutator phenotype (less than fivefold increase in mutation frequencies compared to the wild-type) (Wiegand *et al.*, 2008). An additional gene entitled *pfpI* was also shown to play an anti-mutator role providing general stress protection (Rodriguez-Rojas & Blazquez, 2009).

It has been shown that the prevalence of *P. aeruginosa* mutators increases with time during chronic lung infection due to positive selection of the mutators in the CF lung (Ciofu *et al.*, 2005; Hogardt *et al.*, 2007; Mena *et al.*, 2008)

Association between strong mutators and development of resistance to antibiotics has been reported on several occasions (Oliver *et al.*, 2000; Ciofu *et al.*, 2005; Henrichfreise *et al.*, 2007; Ferroni *et al.*, 2009) and we have recently shown that even small increases in the mutation frequencies, less than 20 times the mutation frequency of PAO1, can represent an advantage for the adaptation of the strains to the antibiotic treatment (Mandsberg *et al.*, 2009).

Inactivation of the MMR system was shown to favour the emergence *in vitro* of phenotypic variants considered typical markers of CF lung infection such as mucoidy due to mutations in *mucA* and loss of quorum sensing due to mutations in *lasR* (Smania *et al.*, 2004). However, the time sequence in which the hypermutable, mucoid and quorum-sensing negative phenotypes emerge during chronic lung infection has not been investigated before in clinical CF *P. aeruginosa* isolates.

In this study, we took advantage of the possibility of following the genetic adaptation of different clonal lineages by characterizing the accumulation of loss-of-function mutations in *mucA*, *lasR* and six anti-mutator genes in longitudinal samples from 10 CF patients.

The sequence variability of the anti-mutator genes belonging to the MMR system (*mutS*, *mutL* and *uvrD*) and GO system (*mutT*, *mutY* and *mutM*) as well as of *lasR* and *mucA* were investigated in 70 sequential isolates with weak or strong mutator phenotype and in non-mutators collected at time points from 5 up to more than 25 years of chronic lung infection (one to three isolates per time point). The significance of the identified mutations for the anti-mutator gene functionality was investigated by complementation with plasmids containing the respective wild-type allele.

METHODS

CF patients, bacterial strains and growth conditions. In the Copenhagen CF Centre, all patients are monitored at monthly visits by evaluation of their clinical status, pulmonary function and microbiology of the lower airways secretions. Chronic pulmonary infection is defined as the persistent presence of *P. aeruginosa* in the sputum for 6 consecutive months, or less if the persistence is combined with the presence of two or more precipitating antibodies against *P. aeruginosa*. Sputum samples obtained by expectoration or endolaryngeal suction are Gram-stained and examined under the microscope to confirm the origin from the lower airways. Since 1976 regular 14 day courses of intensive intravenous treatment (combination of a β-lactam with an aminoglycoside) have been administered every third month to repeatedly restore the lung function and, since 1984, colistin inhalations supplemented if necessary with oral ciprofloxacin have been administered between the intravenous courses.

P. aeruginosa isolates have been collected from sputum of CF patients since 1973 and stored in nutrient broth containing 5 % glycerol at −80 °C. The selection criteria were phenotypic (mucoid and non-mucoid) and different patterns of resistance to antibiotics, as determined on the basis of direct sensitivity testing of isolates from plated sputum. Sequential isolates (70 isolates) from 10 CF patients with chronic *P. aeruginosa* lung infection were included in this study. Previous measurements of mutation frequencies identified these 10 patients as harbouring mutator strains in their lung in the late stages of the chronic infection. For practical purposes the duration of the chronic lung infection was expressed in five 5-year periods from 5–10 years (11 isolates), 10–15 years (12 isolates), 15–20 years (17 isolates), 20–25 years (15 isolates) and more than 25 years (15 isolates) of chronic infection.

Genotyping by PFGE. All isolates were genotyped by PFGE as described previously using *Spe*I enzyme (Ojeniyi *et al.*, 1991; Romling & Tümmler, 2000). After PFGE, the band patterns were visualized by ethidium bromide staining and then photographed (GelDoc imaging system, Bio-Rad). The patterns were analysed by Fingerprinting II software (Bio-Rad). The clonal relatedness of the individual pairs of mucoid and non-mucoid *P. aeruginosa* was confirmed according to Tenover *et al.* (1995). Isolates with PFGE patterns that differ from each other by two to three bands were considered clonally related, as this pattern is consistent with a single genetic event, i.e. a point mutation or an insertion or deletion of the DNA. Isolates with PFGE patterns that differed by more than three bands were considered to belong to different strains.

Measurement of mutation frequencies of *P. aeruginosa* isolates. To determine the mutation frequencies after exposure to rifampicin and streptomycin, the bacterial isolates were grown overnight in 20 ml Luria–Bertani (LB) medium, centrifuged at 3000 r.p.m. for 10 min, and resuspended in 1 ml LB medium. A 100 µl volume of 0, 10^{-1}, 10^{-2} or 10^{-3} dilutions was plated on LB plates containing 300 µg rifampicin ml^{-1} and on LB plates containing 500 µg streptomycin ml^{-1}. A 100 µl volume of 10^{-7} to 10^{-10} dilutions was plated on LB plates, and the numbers of c.f.u. were counted after incubation at 37 °C for 48 h. The mutation frequencies are the mean values from two measurements.

According to the mutation frequencies on rifampicin plates, the isolates were grouped into strong mutators (Hp) [20 times the frequency of mutation of PAO1 ($\geqslant 2\times10^{-7}$)], weak mutators (wHp) ($<2\times10^{-7}$ and $\geqslant 2\times10^{-8}$), or non-mutators (nhp) ($<2\times10^{-8}$).

Determination of antibiotic resistance. The MICs of ceftazidime, meropenem, tobramycin, ciprofloxacin and colistin against the 70 longitudinal *P. aeruginosa* isolates were determined by the agar plate dilution method as previously described (Ciofu *et al.*, 1996) or by E-test (AB Biodisk), according to the manufacturer's instructions.

Sequence analysis of *mutS*, *mutL*, *uvrD*, *mutY*, *mutM* and *mutT*. The *mutS*, *mutL*, *uvrD*, *mutY*, *mutM* and *mutT* genes from all the isolates included in the study were PCR amplified using the primer sets presented in Supplementary Table S1 (available with the online version of this paper). After purification (Promega Wisart purification kit) the PCR products were fully sequenced using the sequencing primers presented in Supplementary Table S1. The sequencing was done on a Macrogen automatic DNA sequencer ABI3700. The number of reads was between two and four for each gene of each strain. The sequence results were compared with the strain PAO1 sequence (http://www.pseudomonas.com) with DNASIS Max version 2.0 (Hitachi Software Engineering), in order to determine the occurrence of sequence variants in the six anti-mutator genes analysed.

Sequence analysis of *lasR* and *mucA*. *lasR* and *mucA* from all the isolates included in the study were PCR amplified. The primers used for *lasR* amplification and sequencing were *lasR* start (5′–3′) ATGGCCTTGGTTGACGGTT and *lasR* stop (5′–3′) GCAAGATCAGAGAGTAATAAGACCCA.

The primers used for *mucA* amplification and sequencing were mucA-1 (5′–3′) CTCTGCAGCCTTTGTTGCGAGAAG; mucA-1 rev (5′–3′) CTGCCAAGCAAAAGCAACAGGGAGG; mucA-2 (5′–3′) GTGCGTCTGTACAACCAGAACGACG; and mucA-2rev (5′–3′) GTCGTTCTGGTTGTACAGACGCACG.

After purification (Promega Wisart purification kit) the PCR products were sequenced on a Macrogen automatic DNA sequencer ABI3700. The number of reads was between two and four for each gene of each strain. The sequence results were compared with the strain PAO1 sequence (http://www.pseudomonas.com) with DNASIS Max version 2.0 (Hitachi Software Engineering), in order to determine the occurrence of sequence variants in the genes analysed.

Construction of complementation plasmids. The *P. aeruginosa*–*Escherichia coli* shuttle vector pUCP26 (West *et al.*, 1994) was used for the construction of recombinant plasmids containing the PAO1 *mutL*, *uvrD*, *mutT*, *mutY* and *mutM* genes under the control of the plasmid-borne *lac* promoter. The plasmids were constructed by amplifying *mutL*, *uvrD*, *mutT*, *mutY* and *mutM* from PAO1 templates with primers provided with restrictions sites matching those in the pUCP26 multi-cloning-site and a Shine–Dalgarno motif, as previously described (Mandsberg *et al.*, 2009). The resulting recombinant plasmids were pLM103 (*mutL*), pLM104 (*uvrD*), pLM101 (*mutT*), pLM100 (*mutY*) and pLM102 (*mutM*).

Complementation with MMR or GO genes. Plasmid pUCPMS harbouring the wild-type *mutS* gene from PAO1 (Oliver *et al.*, 2004), pLM103 (*mutL*), pLM104 (*uvrD*), pLM101 (*mutT*), pLM100 (*mutY*) and pLM102 (*mutM*) were electroporated into clinical isolates with detected changes in the sequence of the respective genes. The transformed bacteria were inoculated on LB agar containing tetracycline (gentamicin for pUCPMS) at concentrations able to inhibit the growth of the CF *P. aeruginosa* isolates. As control, the empty plasmid pUCP26 was electroporated into the clinical isolates. Plasmid DNA extraction of transformed colonies confirmed the presence of plasmids, and mutation frequencies were measured.

Statistical analysis. The description and analysis of data were carried out by using Statview 5 software. The data are given as geometric means for mutation frequencies. Unpaired *t*-test was used to compare mutation frequencies of the *P. aeruginosa* isolates collected in the five different periods of chronic lung infection after log-transformation of the data in order to normalize the distribution.

RESULTS

Clonal distribution of the *P. aeruginosa* isolates

The PFGE showed that six different clones of *P. aeruginosa* were represented among the 70 isolates from the 10 Danish CF patients. The patients maintained the same bacterial clones in their lungs during the long period of chronic lung infection (data not shown). Two previously reported dominant clones (DK-1 or 'r' and DK-2 or 'b') (Ojeniyi *et al.*, 1993; Jelsbak *et al.*, 2007; Ciofu *et al.*, 2008) were shared by several patients (CF 2, CF 5, CF 7 and CF 10 harboured DK-1 while CF 3, CF 4 and CF 9 harboured DK-2). Unique clones (A, B, C and D) were harboured by the last three patients although one patient had simultaneously

Table 1. Summary of the type of mutations identified in anti-mutator genes and in *mucA* and *lasR* genes of sequential *P. aeruginosa* isolates with strong (Hp), weak (wHp) or non-mutator (nhp) phenotype in 10 CF patients with chronic lung infection

The isolates are identified by a number representing the number of years the CF patient had been chronically infected at the time of isolate collection. When both non-mucoid (NM) and mucoid (M) phenotypes were available from the sputum sample, the isolate is identified by a number followed by NM or M. The clone (see text) to which each isolate belongs is also presented. nc, no changes identified in the respective gene sequence, ND, sequence not determined.

CF patient	Isolate[clone]	Mutator phenotype and genotype	Mutations in *mucA* and the respective changes in MucA	Mutations in *lasR* and the respective changes in LasR
CF 1	13^{A}, $15NM^{A}$, 16^{A}	wHp *mutT* Insertion G at 114: stop codon	nc	G264T:Trp88Cys
	$15M^{B}$, $20M^{B}$, $21M^{B}$, $23M^{B}$	nhp, wHp ($20M^{B}$) No mutations	C352T: stop codon	C292T: stop codon
	$20NM^{B}$, $35NM^{B}$	nhp No mutations	Δ105 bp at 306: frameshift	C292T: stop codon
	21 NM^{B}, 23 NM^{B}29 NM^{B} 30 NM_2^{B}	nhp, wHp (23NM) No mutations	nc	C292T: stop codon
	$29M^{B}$, $30M^{B}$, $35M^{B}$	Hp *mutS* Δ15 bp at 1098: frameshift	C352T: stop codon	C292T: stop codon
	$30NM\_1^{A}$	wHp *mutT* Insertion G at 114: frameshift	nc	nc
CF 2	16^{DK-1}	nhp No mutations	C349T: stop codon	nc
	22^{DK-1}	Hp *mutS* ΔA at 2429: frameshift	nc	C668T: Ser223Phe
	24^{DK-1}	Hp *mutLYM* polymorphism	nc	C538T: Arg180Trp
	$27M^{DK-1}$	Hp *mutS* ΔA at 2429: frameshift	C349T: stop codon	A293G: Gln98Arg
	$27NM^{DK-1}$	Hp *mutLYM* polymorphism	nc	C538T:Arg180Trp
	29^{DK-1}	Hp *mutSLYM* polymorphism	nc	C538T:Arg180Trp
	$33M^{DK-1}$	Hp *mutS* ΔA at 2429: frameshift	C349T: stop codon	nc
	$33NM^{DK-1}$	*mutLYM* polymorphism	nc	C538T:Arg180Trp
CF 3	4^{DK-2}	nhp No mutations	ΔG430: frameshift	Not amplified
	5^{DK-2}	nhp No mutations	ΔG430: frameshift	Not amplified
	11^{DK-2}, 12^{DK-2}, 14^{DK-2}, $15NM^{DK-2}$, $15M^{DK-2}$	Hp *mutS* ΔA at 2210: frameshift	ΔG430: frameshift	Not amplified
CF 4	5^{DK-2}, 12^{DK-2}	nhp No mutations	ΔG430: frameshift	Not amplified
	15^{DK-2}, $18M^{DK-2}$, $18NM^{DK-2}$, $20M^{DK-2}$, $20NM^{DK-2}$	Hp *mutS* G1567A: Glu522Lys	ΔG430: frameshift	Not amplified
CF 5	10^{DK-1}	nhp No mutations	nc	ND

Table 1. cont.

CF patient	Isolate[clone]	Mutator phenotype and genotype	Mutations in *mucA* and the respective changes in MucA	Mutations in *lasR* and the respective changes in LasR
	17^{DK-1}	nhp No mutations	nc*	nc
	20^{DK-1}	Hp *mutS* Insertion A at 2355: frameshift	ΔG430: frameshift	Δ5 bp at 187: frameshift
	25^{DK-1}	wHp *uvrD* C1645T: Ala548Val	nc	T530C: Leu177Pro
CF 6	9^{C}, 10^{C}	wHp (9^{C}), nhp *mutM* G196A: Ala65Thr	nc	Δ13 bp at 666: frameshift
	21^{C}, 25^{C}, 27^{C}	Hp *mutL* ΔG at 438: frameshift	nc	Δ13 bp at 666: frameshift
CF 7	16^{DK-1}	nhp No mutations	Δ GCA at 347: in-frame Gly deletion	nc
	$18M^{DK-1}$	nhp No mutations	nc	nc
	$18NM^{DK-1}$, 21^{DK-1}	Hp *mutLYM* polymorphism	nc	C538T: Arg180Trp
	23^{DK-1}	nhp No mutations	C349T: stop codon	nc
CF 8	5^{D}	nhp No mutations	nc	ND
	6^{D}	nhp No mutations	nc	G313A: Ala105Thr
	8^{D}, $9M^{D}$	Hp *mutS* ΔGC at 1554: frameshift	ΔG430: frameshift	G313A: Ala105Thr
	$9NM^{D}$	Hp *mutS* ΔGC at 1554: frameshift	nc	G313A: Ala105Thr
CF 9	14^{DK-2}	nhp No mutations	ΔG430: frameshift	Not amplified
	$19M^{DK-2}$	nhp No mutations	C349T: stop codon	Not amplified
	$19NM^{DK-1}$	Hp *mutS* G1567A: Glu522Lys	C349T: stop codon	nc
	24_1^{DK2}	Hp *mutS* G1567A: Glu522Lys	ΔG430: frameshift	Not amplified
	24_2^{DK-2}	Hp *mutL* Insertion 5 bp at 1246: frameshift	ΔG430: frameshift	Not amplified
	27^{DK-2}	Hp *mutS* G1567A: Glu522Lys	ΔG430: frameshift	Not amplified
CF 10	$20M^{DK-1}$	nhp No mutations	C349T: stop codon	nc
	$20NM^{DK-1}$	Hp *mutL* Large insertion at 1882	nc	T227C: Val76Ala
	$28M^{DK-1}$	nhp No mutations	C349T: stop codon	nc

Table 1. cont.

CF patient	Isolate[clone]	Mutator phenotype and genotype	Mutations in *mucA* and the respective changes in MucA	Mutations in *lasR* and the respective changes in LasR
	28NM[DK-1]	Hp *mutL* Large insertion at 1882	C349T: stop codon	T227C: Val76Ala

*In this isolate the sequence of *algT* showed ΔGCACA at position 89 leading to a frameshift, showing that mutation had occurred in the *algT* operon.

two different clones (A and B) in the lung during the chronic lung infection (Table 1, Supplementary Table S2).

Mutation frequency of CF *P. aeruginosa* sequential isolates

Among the 70 isolates, 54 % (38) had strong mutator phenotype (Hp), 11 % (8) had weak mutator phenotype (wHp) and 34 % (24) had non-mutator phenotype (nhp). Isolates with Hp, wHp or nhp were present in all the five time-groups of isolates, showing that in the CF lungs different bacterial subpopulations may co-exist over a long period (Fig. 1).

The mutation frequencies (geometric mean) to rifampicin and streptomycin resistance of the isolates collected in the late stages of the chronic infection (the group 'more than 25 years') were 5.4×10^{-7} and 1.45×10^{-6}, respectively, and these were significantly higher ($P=0.03$) than the mutation frequencies of isolates collected in the initial stages of the chronic infection (the group '5–10 years'), which were 5.1×10^{-8} and 1.54×10^{-7}, respectively.

In accordance with previous studies, these data suggest that in the course of chronic infection, an accumulation of mutants occurs due to bacterial replication (Oliver *et al.*, 2004; Ciofu *et al.*, 2005; Mena *et al.*, 2008).

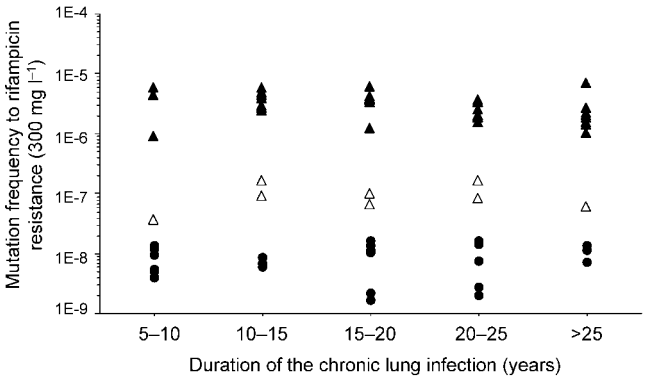


Fig. 1. Mutation frequencies after exposure to rifampicin in 70 *P. aeruginosa* isolates collected in five periods of chronic lung infection (5–10, 10–15, 15–20, 20–25, and more than 25 years) from 10 patients with CF. The results for strong mutators (Hp; ▲), weak mutators (wHp; △) and non-hypermutators (nhp; ●) are presented.

Antibiotic susceptibility

The MICs of ceftazidime and ciprofloxacin [median (range) μg ml^{-1}] for isolates collected in the first 5–10 year period of chronic lung infection [6.2 (1–100) and 0.25 (0.1–3.1)] were significantly lower compared to those found in isolates collected in the periods 10–15 years [400 (2–400) and 6 (0.03–50); $P=0.0004$ and non-significant, respectively], 15–20 years [200 (1.6–400) and 3.1 (0.4–25); $P=0.004$ and $P=0.01$, respectively], 20−25 years [57 (0.1–400) and 9.3 (0.5–25); $P=0.01$ and $P=0.006$, respectively] and more than 25 years [100 (0.75–400) and 3.1 (0.5–12.5); $P=0.01$ and $P=0.005$, respectively] of chronic lung infection. These results are in agreement with previous studies (Ciofu *et al.*, 1994), showing development of resistance to antibiotics in *P. aeruginosa* isolates during chronic lung infection.

The MICs of ceftazidime, meropenem, tobramycin, ciprofloxacin and colistin for the Hp, wHp and nhp *P. aeruginosa* isolates are presented in Table 2. The Hp isolates were significantly more resistant to all the tested antibiotics, except colistin, than the nhp isolates. Interestingly, the MIC of tobramycin was significantly higher for the wHp isolates compared to the nhp isolates.

Sequences of anti-mutator genes in consecutive CF *P. aeruginosa* isolates from different time points of the chronic lung infection

The types of mutation identified in the different bacterial clones infecting the 10 CF patients are summarized in Table 1 and described in detail in Supplementary Table S2.

Mutations in *mutS* were found in 68 % (26 isolates belonging to seven CF patients) and in *mutL* in 16 % (six isolates from three CF patients) of the 38 Hp isolates. Complementation with the wild-type genes confirmed that the mutations identified were responsible for the mutator phenotype (Supplementary Table S2). However, in 16 % (six isolates from two patients) the genetic background of the Hp remains unclear. Although these six isolates showed simultaneous mutations leading to amino acid changes in *mutL*, *mutY* and *mutM*, the complementation studies with

Table 2. MICs of ceftazidime, meropenem, tobramycin, ciprofloxacin and colistin [median, (ranges)] in sequential CF isolates with strong (Hp), weak (wHp) or non-mutator (nhp) phenotypes

The MICs were not determined in three isolates.

Phenotype (number)	MIC (μg ml^{-1})				
	Ceftazidime	Meropenem	Tobramycin	Ciprofloxacin	Colistin
Hp (36)	200 (0.75–400)	25 (0.2–400)	10.2 (0.4–100)	6.2 (0.25–50)	0.8 (0–512)
wHp (8)	25 (3–400)	2.8 (0.2–32)	10.2 (1–98)	0.9 (0.03–12.5)	0.65 (0.1–1.6)
nhp (23)	25* (0.1–400)	1.6* (0.02–100)	3.1*† (0.1–12.5)	1.6* (0.1–12.5)	1.6 (0.06–3.1)

*$P<0.05$ compared to Hp isolates.
†$P<0.05$ compared to wHp isolates.

each of the wild-type genes could not confirm these mutations as the cause of high mutation frequencies in these isolates. Analysis of the promoter regions of all the anti-mutator genes showed no differences between the sequences of the six Hp isolates and the corresponding early nhp isolates from the two patients.

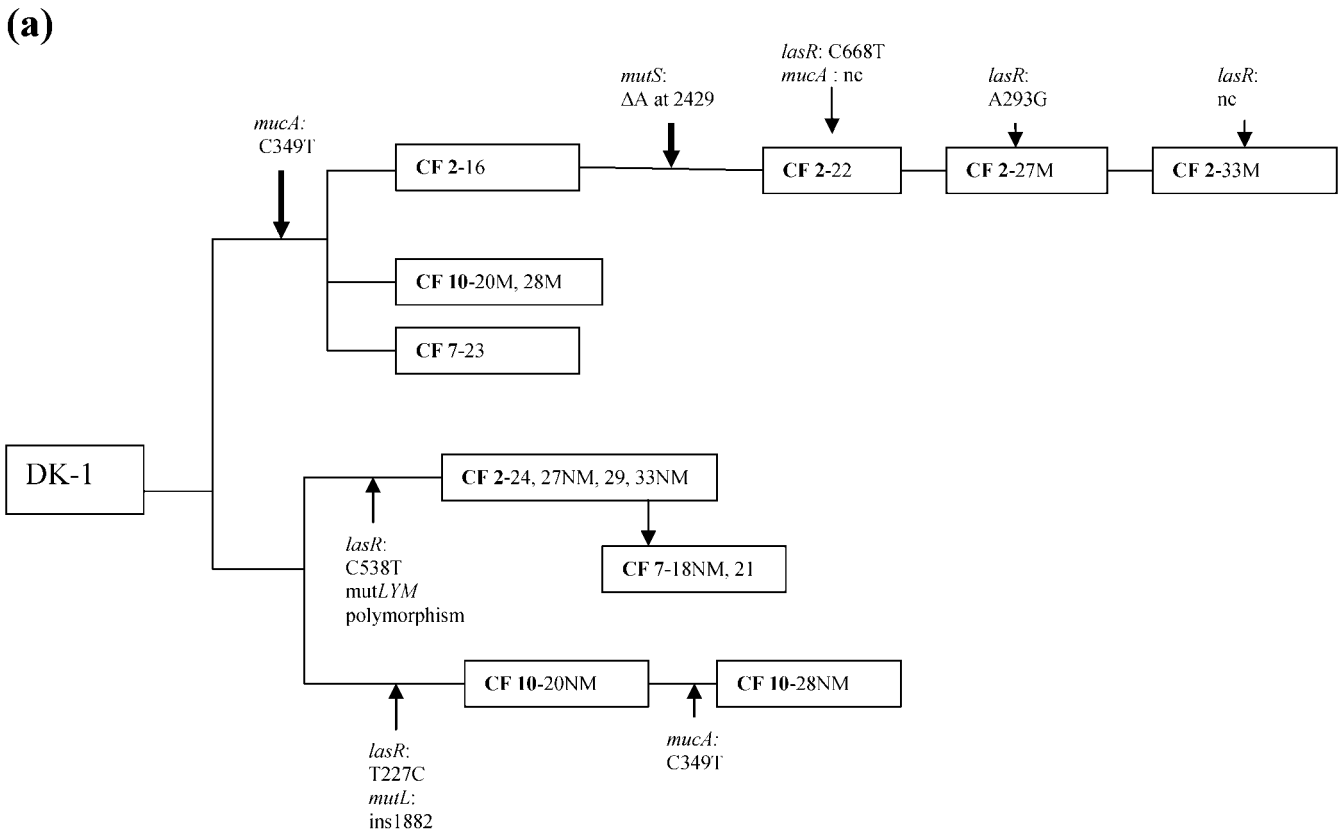
Four out of eight isolates with wHp showed mutations in *mutT* and complementation with the wild-type *mutT* reversed the mutator phenotype. All these isolates were collected from one CF patient. Mutations in *uvrD* and *mutM* leading to changes in the amino acid sequences (Ala548Val in UvrD) and (Ala65Thr in MutM) were identified in two other wHp isolates from two other patients. However, complementation with the respective wild-type genes failed to reverse the wHp phenotype, and the *mutM* mutation was found in a nhp isolate as well, making unclear the role of these mutations for the acquisition of the wHp. In the remaining two isolates with wHp phenotype, no mutations were identified in the six genes that were investigated.

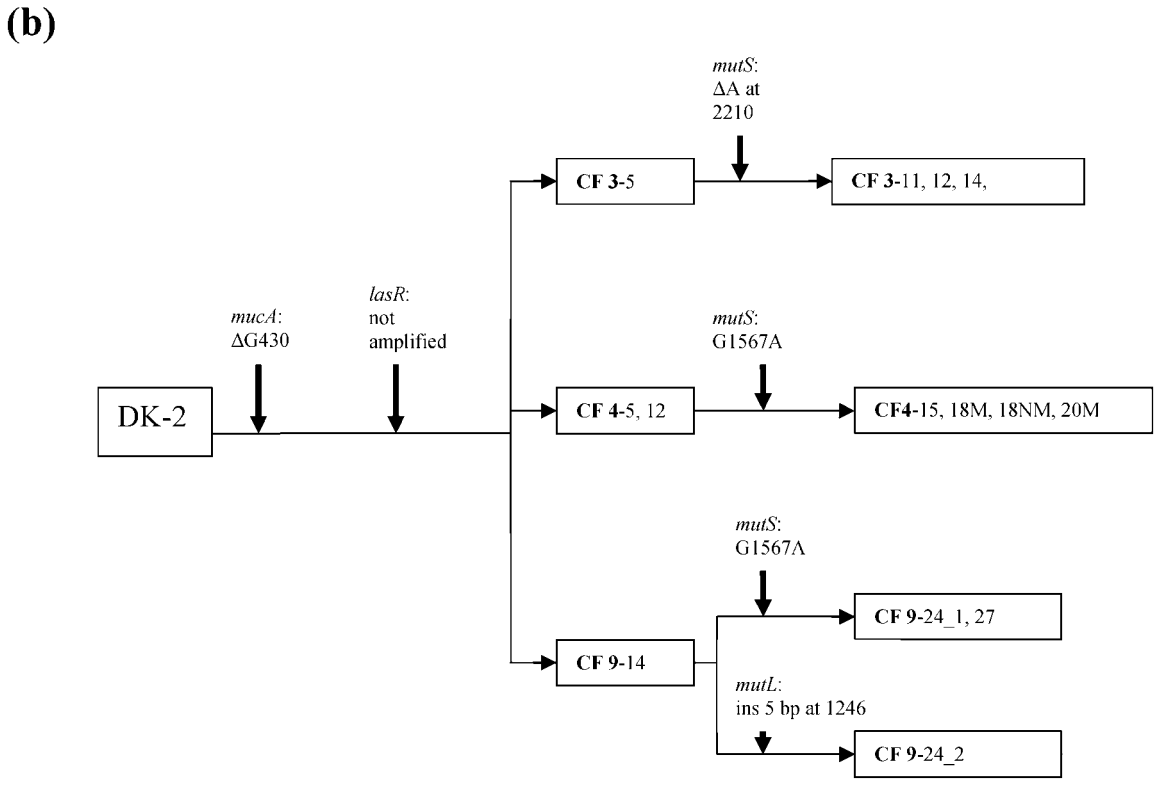
In 19 out of 24 isolates with nhp, no changes were found in the six anti-mutator genes that have been investigated (except the sequence polymorphisms found in *uvrD* and *mutT* that are mentioned below). However, a 10 bp deletion leading to a frameshift in *mutY* and mutations in *mutM* leading to Ala65Thr in the encoded protein were identified in nhp isolates from two patients. Simultaneous mutations in *mutS*, *mutL* and *mutY* causing amino acid changes in the encoded proteins were identified in two nhp isolates of CF 8 (Supplementary Table S2), suggesting that the respective mutations did not influence the functionality of the encoded proteins.

The types of mutations identified were not associated to a specific bacterial clone. Although patients CF 2, CF 5, CF 7 and CF 10 harboured clone DK-1 in their lungs, whereas CF 3, CF 4 and CF 9 harboured DK-2, the sequence variability of the mutator genes was unique to each patient. The same type of mutation was present in sequential isolates, showing that the mutator phenotype occurred after the patients had been infected with these dominant clones and had not been transmitted to other patients. However, one exception is the G1567A mutation found in *mutS* leading to change of the Glu to Lys at position 522. This mutation was found in mutator isolates belonging to the DK-2 clone in patients CF 9 and CF 4.

Patient CF 1 had *P. aeruginosa* belonging to clone A and B in the lung during the chronic infection. While mutators of clone A had a wHp phenotype due to loss-of-function mutation in *mutT*, clone B mutators expressed a Hp phenotype due to loss-of-function mutation in *mutS*. Patient CF 2 had mutators with mucoid phenotype due to mutations in *mutS*, while the non-mucoid mutators had mutations in unidentified loci. In patient CF 9, inactivation of *mutS* or *mutL* was found in two isolates with Hp phenotype collected from the same sputum sample. All these examples show that mutators with different genetic backgrounds are maintained during the chronic infection, suggesting that these mutator lineages are not competing with each other in the CF lung.

Fig. 2. Spread of DK-1 (a) and DK-2 (b) clones among CF patients in the Copenhagen CF centre and acquisitions of mutations in *lasR*, *mucA* and anti-mutator genes. (a) For the DK-1 clone, a possible scenario is that two lineages, a mucoid (*mucA*: C349T) and a non-mucoid (*mucA* wild-type) spread separately. The mucoid lineage infected CF 2 (CF2-16), CF 7 (CF7-23) and CF 10 (CF10-20M). A cross-infection between patients is also possible. In CF 2, the *mucA*$^-$ isolate (CF2-16) acquired a mutation in *mutS*, which was followed by various mutations in *lasR* in later isolates. In CF 10, the *mucA*$^-$ isolate (CF 10-20M) was maintained unchanged (CF10-28M). The non-mucoid (*mucA*$^+$) lineage infected CF patients before (CF 5, data not shown) or after acquisition of various mutations in *lasR* and *mutL* (CF 2, CF 10). A possible spread of the non-mucoid lineage from patient CF 2 to patient CF 7 is shown. (b) In the DK-2 clone, mutations in both *lasR* and *mucA* occurred before spreading to CF 3, CF 4 and CF 9. Later, various mutations in the anti-mutator genes occurred in the isolates infecting these three patients.





The majority of mutations in *mutS* and *mutL* were deletions or insertions leading to premature stop codons (Table 1). The point mutation G1567A in *mutS* led to an amino acid change Glu522Lys in the core domain III of MutS (Table 1) and the mutator phenotype reverted after complementation with the wild-type gene. As the core domain is connected to the ATPase domain, we suggest that this mutation might have consequences for the conformation of MutS at the level of the ATP-binding site (Lamers *et al.*, 2000).

Sequence polymorphisms were found in both *uvrD* and *mutT*. In *uvrD*, the mutations GT1985AC and A1997G leading to amino acid changes from Ser to Asn and Asn to Ser, respectively, were found in 90 % of the isolates investigated (Supplementary Table S2). This polymorphism has been described previously in *P. aeruginosa* strains with mutator and non-mutator phenotypes isolated at the Hannover CF Clinic (Montanari *et al.*, 2007).

In *mutT*, the point mutation G708T leading to an amino acid change from Glu to Asp at position 236 was associated with clones DK-1, B and D (present in 88 % of the isolates belonging to these three clones) while C416T leading to amino acid change Ser to Leu at position 139 was associated with clone DK-2 (100 % of the DK-2 isolates) (Supplementary Table S2). These polymorphisms in *uvrD* and *mutT* were present in isolates with mutator and non-mutator phenotypes, suggesting that they are not affecting the mutation frequency of the isolates.

In a large number of clinical isolates (CF1_29M, CF1_30NM-1, CF2_24, CF2_27NM, CF2_29, CF2_33NM, CF5_20, CF6_21, CF6_27, CF7_18NM, CF7_21, CF8_5, CF 8_6, CF8_8, CF8_9M, CF8_13, CF8_16, CF9_24-1, CF9_24-2) mutations in several anti-mutator genes, such as *mutS*, *mutL*, *mutY* and *mutM*, were identified (Supplementary Table S2). Although complementation with single wild-type genes of the MMR system reduced the frequency of mutations in these isolates, we cannot exclude that the mutations identified in the genes of the GO system also play a role in the occurrence of the mutator phenotype.

Sequence of *mucA* and *lasR* in consecutive CF *P. aeruginosa* isolates from different time points of the chronic lung infection

Inactivation of the MMR system was shown *in vitro* to favour the emergence of *mucA22* and *lasR* mutants (Smania *et al.*, 2004; Lujan *et al.*, 2007; Moyano *et al.*, 2007), and an association between hypermutability and mucoidy has been reported in clinical CF isolates (Waine *et al.*, 2008).

We investigated in our collection of longitudinal *P. aeruginosa* CF isolates at which time point mutations in *mucA* and *lasR* occurred and compared it to the time point when mutations in the anti-mutator genes were identified. Mutations in anti-mutator genes occurred in the chronic CF isolates that already had mutations in *mucA* and/or *lasR*, as shown in Table 1 and Fig. 2.

The mutations identified in *mucA* were C349T and ΔG430 in isolates belonging to clone DK-1 and DK-2, respectively (Fig. 2a, b). In addition, *lasR* could not be amplified in either of the DK-2 isolates, suggesting the probable deletion of this gene in the DK-2 clone (in accordance with unpublished results of Lei Yang, Infection Microbiology Group, Technical University of Denmark). These patterns of mutations in *mucA* and *lasR* suggest that the spread of these two dominant clones among CF patients in Copenhagen occurred after the acquisition of the respective *mucA* and/or *lasR* mutations and one may hypothesize that these mutations are not affecting the infectivity of *P. aeruginosa* in new environments (Fig. 2a, b). In contrast, the mutations encountered in the anti-mutator genes in isolates of DK-1 and DK-2 varied among patients, suggesting that the evolution of mutators occurred later during chronic infection after the spread of these two dominant clones (Fig. 2a, b). The similar evolutionary pathways encountered in the different CF patients may be considered an example of adaptive radiation, where niche specialists occur in the different compartments of the CF lung.

DISCUSSION

In the present collection of *P. aeruginosa* isolates from chronically infected CF patients, mutations in both *mucA* and *lasR* preceded the occurrence of mutations in the anti-mutator genes, suggesting that the selection pressures for maintenance of mucoidy and *lasR*-deficient phenotypes are already acting in the initial phases of the chronic lung infection. Previous data (Waine *et al.*, 2008) suggested an association between hypermutability and mucoidy and the authors even proposed a possible interaction between the two phenotypes due to the higher rates of mutations in hypermutators that would make more likely the acquisition of mutations resulting in the mucoid phenotype. Although this is an attractive hypothesis that fits with some *in vitro* observations (Lujan *et al.*, 2007; Moyano *et al.*, 2007), we could not confirm it by the genetic analysis of our collection of *P. aeruginosa* CF isolates from the late phases of the chronic lung infection.

Our data show that mucoid isolates as well as *lasR* quorum-sensing-deficient isolates are present in the initial phase of the infection while mutator isolates occur in the later phases of the infection; this is in accordance with earlier reports (Pedersen *et al.*, 1992; D'Argenio *et al.*, 2007). The occurrence of *mucA* and *lasR* mutation in bacteria that initially were Hp but which subsequently reverted to the nhp phenotype due to the disadvantage of the Hp in competition with other strains is a possible scenario that cannot be ruled out by our data. However, competition among different strains is less likely in the strongly compartmentalized CF lung.

Mucoid variants were reported to arise with a frequency of 1 in 10^7 (Govan & Fyfe, 1978). *P. aeruginosa* is present in

very high numbers in the CF lung, 10^7–10^{10} c.f.u. ml^{-1}, which can readily explain the possibility for alginate producers to emerge in the absence of a strong mutator phenotype. In addition, we have shown that exposure of the bacteria to activated polymorphonuclear leukocytes (PMNs) creates *mucA* mutations in the absence of hypermutability and the inflammatory response is an early event during infection (Mathee *et al.*, 1999). It has also been shown that spontaneous *lasR* mutants occur with a high frequency, making the involvement of hypermutability unlikely (D'Argenio *et al.*, 2007; Sandoz *et al.*, 2007). Subsequently, both mucoid and LasR-deficient isolates will be selected in the CF lung. The mucoid isolates will be selected because of a better survival due to the protective effects of alginate against innate immune factors and reactive oxygen species (Govan & Nelson, 1993) and the *lasR* mutants will be selected due to their growth advantage with particular carbon and nitrogen sources, including amino acids (D'Argenio *et al.*, 2007).

We provide evidence that accumulation of mutators in the CF lung is associated especially with multi-drug resistance development, thus suggesting that the intensive antibiotic treatment is one of the main selective pressures for the maintenance and amplification of mutators, a hypothesis that is supported by several publications (Oliver *et al.*, 2000; Ciofu *et al.*, 2005; Ferroni *et al.*, 2009).

The differences in the antibiotic treatment strategies between the CF centres, as well as sample bias in the Waine study due to an increased number of samples from patients harbouring mucoid isolates, might explain the different results reported previously (Waine *et al.*, 2008).

Bacterial subpopulations with nhp, wHp or Hp phenotypes as well as Hp with different genetic backgrounds were found to co-exist in the CF lung during long periods of chronic infection, in accordance with previous reports (Hogardt *et al.*, 2007; Mena *et al.*, 2008). This suggests a lack of competition between the various mutator subpopulations due to the strong compartmentalization and low migration rates within and between the lungs of CF patients (Denamur *et al.*, 2005). The different mutator subpopulations are probably living in different focal regions (de Jong *et al.*, 2004) or niches in the CF lung (Bjarnsholt *et al.*, 2009). Persistence of the strong mutators in spite of the cost of high mutation rates for long-term adaptation suggests that Hp are niche specialists (Giraud *et al.*, 2001).

Our data show evidence of transmission among CF patients of *P. aeruginosa* with mutations in *mucA* or *lasR* but no evidence of transmission of mutators, confirming the reduced fitness of mutators for new environments. Studies in animal models have also shown that mutators have reduced potential for colonization of new environments and therefore strain transmissibility (Mena *et al.*, 2007). It has been shown previously that mutators adapted to the CF lung lose their virulence and are impaired by deleterious mutations when they have to adapt to secondary environments (Montanari *et al.*, 2007).

Our sequencing strategy allowed us to find loss-of-function mutations in genes involved in the repair of oxidative damage of the DNA in CF *P. aeruginosa* isolates, although it has been suggested that selection of this type of mutants will not take place in the CF lung due to the reduced oxygen tension in the CF sputum (Wiegand *et al.*, 2008).

However, we have recently shown that the anoxic conditions in the sputum are due to the consumption of oxygen by the large number of PMNs which are liberating oxygen radicals (Kolpen *et al.*, 2010). We have previously shown that activated PMNs represent an important source of oxidative stress for the bacterial DNA (Ciofu *et al.*, 2005).

In addition to the increased oxidative burden, *P. aeruginosa* in the CF lung are probably also poorly protected against oxidative stress due to decreased catalase production in *lasR* mutants and during the biofilm mode of growth (Driffield *et al.*, 2008).

Therefore, we suggest that a selection pressure for the occurrence of GO mutants does exist in the CF lung, in spite of the growth in sputum at low oxygen tension.

The prevalence of strong mutators due to mutations in MMR genes in the present and previous studies (Oliver *et al.*, 2002; Mena *et al.*, 2008) is probably due to the bias introduced by the isolation criteria of the *P. aeruginosa* strains from the sputum of CF patients, which include resistance to antibiotics. Analysis of mutation frequencies on a large number of *P. aeruginosa* colonies grown from the sputum would probably show a higher heterogeneity of mutators in the bacterial population.

Our strain collection was represented by *P. aeruginosa* isolates from the late stages of the chronic lung infection (from 4 up to 35 years of chronic lung infection) but did not include isolates from the phase of intermittent colonization or from the very early phases of the chronic lung infection. It has been previously published that 6 % of the environmental isolates were weak mutators and 10 % of early isolates were strong or weak mutators, but only the sequence of *mutS* was analysed in these isolates. Based on our present results, it would be interesting to investigate in a future prospective study the GO system genes in environmental or early *P. aeruginosa* isolates with weak mutator phenotype.

Mutations in several anti-mutator genes were found in a large number of isolates, suggesting the possibility of their synergistic effect on mutability. Simultaneous inactivation of both *mutY* and *mutM* had a synergistic effect in *E. coli* (Michaels *et al.*, 1992; Miller, 1996) and similar results have been obtained in *P. aeruginosa* in our laboratory (unpublished results). A mutator cascade, in which one type of mutator (*mutT*) generates a second mutator (*mutHLS*) that then allows stepwise frameshift mutations, has been described in *E. coli* (Miller *et al.*, 1999).

The types of mutation identified in the anti-mutator genes were very different from clone to clone and from CF patient to CF patient, suggesting that there are no hot-spot regions in the DNA sequence of these genes prone to mutability, such as those identified in *mucA* in mucoid CF isolates (Ciofu *et al.*, 2008).

In conclusion, our study shows that mutators with heterogeneous genetic background occur in isolates that have already acquired mutations in *mucA* or *lasR* and that these mutators are maintained in the lung during the chronic infection, suggesting parallel evolution of the different mutants in the various niches in the CF lung.

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