Sequence diversity of the *mucABD* locus in *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis

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The *mucA* gene of the *muc* operon, which is instrumental in the control of the biosynthesis of the exopolysaccharide alginate, is a hotspot of mutation in *Pseudomonas aeruginosa*, a micro-organism that chronically colonizes the airways of individuals with cystic fibrosis (CF). The *mucA*, *mucB* and *mucD* genes were sequenced in nine environmental isolates from aquatic habitats, and in 37 *P. aeruginosa* strains isolated from 10 patients with CF, at onset or at a late stage of chronic airway colonization, in order to elucidate whether there was any association between mutation and background genotype. The 61 identified single nucleotide polymorphisms (SNPs) segregated into 18 *mucABD* genotypes. Acquired and *de novo* stop *mucA* mutations were present in 14 isolates (38 %) of five *mucABD* genotypes. $\Delta G430$ was the most frequent and recurrent *mucA* mutation detected in four genotypes. The classification of strains by *mucABD* genotype was generally concordant with that by genome-wide *SpeI* fragment pattern or multilocus SNP genotypes. The exceptions point to intragenic mosaicism and interclonal recombination as major forces for intraclonal evolution at the *mucABD* locus.

INTRODUCTION

The metabolically versatile *Pseudomonas aeruginosa* is present in soil and aquatic habitats, but it is also an important opportunistic pathogen for humans, animals and plants. Typing of strain collections by single nucleotide polymorphisms (SNPs), DNA fragment length polymorphisms and phenotypic traits indicates that the current *P. aeruginosa* population is in linkage equilibrium, and consists of a network of equivalent genotypes (termed clones), whereby a subset of clones is overrepresented due to epidemic spread (Curran *et al.*, 2004; Morales *et al.*, 2004; Pirnay *et al.*, 2002a, b).

In *P. aeruginosa*, interclonal and intraclonal diversity have been reported to result from the acquisition or loss of genomic islets or islands, genome rearrangements, and recombination or point mutation (reviewed by Tümmler, 2006). Mosaic genes are a further source of genetic diversity. They contain regions with different degrees of relatedness which can be explained by recombination of two distinct alleles as a result of gene transfer events (Hakenbeck, 1998).

Evidence for a mosaic gene structure has been drawn from SNP haplotype (Spangenberg *et al.*, 1998; Kiewitz & Tümmler, 2000) or the detection of cassettes (Spangenberg *et al.*, 1996). Our current knowledge about mosaic genes in *P. aeruginosa* is restricted to the pyoverdine biosynthesis locus (Smith *et al.*, 2006), *ampC* (PA4110) (Spangenberg *et al.*, 1998), *fleP* (PA1096) (Arora *et al.*, 2001), *fliC* (PA1092) (Spangenberg *et al.*, 1998) and *oprD* (PA0958) (Pirnay *et al.*, 2002b). In the case of *oprD*, sequence analysis of 55 *P. aeruginosa* isolates, collected over a period of 15 years from various, spatially separated, clinical and environmental habitats, has uncovered a microscale mosaic structure of *oprD*. All sequences fall into three main groups, which differ by 7–9 % of their nucleotides. Several recombinational exchanges of DNA blocks of 100–300 bp lead to a mosaic gene structure and cause further divergence into subgroups.

Most information about the evolution of intraclonal diversity of *P. aeruginosa* has been obtained from retrospective cross-sectional and longitudinal analyses of isolates recovered from the atypical habitat of the lungs of patients with cystic fibrosis (CF) (Ernst *et al.*, 2003; Kresse *et al.*, 2003; Larbig *et al.*, 2002; Smith *et al.*, 2006). A hallmark of

Abbreviations: CF, cystic fibrosis; SNP, single nucleotide polymorphism.

the adaptation of *P. aeruginosa* to the atypical niche of the CF lung is the emergence of mucoid alginate-overexpressing morphotypes that are typically associated with stop mutations in the *mucA* gene (Martin *et al.*, 1993a).

The activation of alginate genes is complex (reviewed by Ramsey & Wozniak, 2005). The *algU* gene, which is essential for alginate production, encodes the alternative sigma factor σ^{22} . MucA is an anti-sigma factor that sequesters σ^{22} (Mathee *et al.*, 1997; Schurr *et al.*, 1996), and MucB is a periplasmic negative regulator (Martin *et al.*, 1993b). MucD is a homologue of the *Escherichia coli* periplasmic serine protease HtrA, and is apparently both a chaperone and a regulator of alginate production (Wood & Ohman, 2006).

This study reports on the sequence diversity of the *mucABD* operon in nine environmental and 37 CF *P. aeruginosa* isolates. The association between *mucABD* SNP haplotype and chromosomal genotype has been inferred from *SpeI* restriction fragment patterns (Römling *et al.*, 1994) and multilocus SNP genotypes (Morales *et al.*, 2004). The CF strains were isolated from 10 patients at onset and at a chronic late stage of airway colonization. In living patients, the late isolates were collected after 13–15 years of continuous chronic airway colonization, and in deceased patients, the last isolates prior to death were studied. Sequence analysis of sequential isolates has so far only been performed on strains from a young CF patient who acquired *P. aeruginosa* during infancy, and who was monitored for a period of 8 years thereafter (Spencer *et al.*, 2003; Smith *et al.*, 2006).

Since *mucA* mutations are common in CF isolates (Martin *et al.*, 1993a, b), the sequence analysis provided the opportunity to investigate the diversity of the genetic background in a mutation hotspot in the *P. aeruginosa* genome. The comparative study of sequential isolates allowed us to differentiate between acquired and *de novo* mutations. Sequence and genotype analysis provided direct evidence for recurrent *mucA* mutations and the generation of mosaic *muc* genes in *P. aeruginosa* that persists in CF lungs.

METHODS

CF patients, bacterial strains and growth conditions. *P. aeruginosa* strains were isolated from 10 pancreatic-insufficient CF patients attending the CF clinic of the Medizinische Hochschule Hannover. Thirty-seven *P. aeruginosa* strains were analysed from sputa or throat swabs from the 10 CF patients (Table 2). Twenty *P. aeruginosa* strains were collected at the onset of chronic colonization. From the five living patients (patients 1–5), eight additional strains were collected 13–15 years later. From the five deceased patients (patients 6–10), the last isolate collected prior to death 5–14 years after acquisition of *P. aeruginosa* was analysed. Strains were stored at -70°C , and were not subcultured. In addition, the completely sequenced strains *P. aeruginosa* PAO1 and PA14, and nine environmental isolates from aquatic habitats (SG17M, PT2, PT6, PT12, PT20, PT22 and DSM939), mushroom (ATCC 33818) and a fuel tank (ATCC 33988) were studied.

Genotyping by PFGE. Samples were analysed as described by Römling *et al.* (1994), with minor modifications. *P. aeruginosa*

strains were grown overnight at 37°C in LB medium, centrifuged for 10 min at 1500 g, and suspended in 75 mM NaCl, 25 mM EDTA, pH 7.4, to a concentration of 5×10^9 cells ml^{-1} . The cell suspension was mixed 1:1 with 2% (w/v) low-melting-point agarose to prepare the agarose plugs. The embedded cells were digested for 48 h at 56°C with proteinase K (1 mg ml^{-1} in 0.5 M EDTA, pH 9.5, 1%, v/v, *N*-lauryl sarcosine). Agarose plugs were equilibrated and stored in 10 mM EDTA, 10 mM Tris/HCl, pH 7.4, at 4°C until used. One-third to one-half of a plug was equilibrated in *SpeI* buffer (50 mM NaCl, 6 mM Tris/HCl, pH 7.5, 10 mM MgCl_2). Digestion was performed overnight at 37°C in 90 μl enzyme buffer with 4 U *SpeI*, 0.1 mg BSA ml^{-1} and 5 mM DTT. Separation of DNA fragments was performed by PFGE in a CHEF-DR III apparatus (Bio-Rad), using a 1.5% (w/v) agarose gel, 0.5 $\times$ Tris/borate/EDTA buffer, and a linear ramping of 8–50 s for 24 h, 12–25 s for 22 h, and 1–14 s for 14 h. DNA fragments were visualized by ethidium bromide staining. Genotypes were defined from the evaluation of *SpeI* fragment patterns, as described by Römling *et al.* (1994).

Multilocus SNP genotyping. Boiled colonies of each strain were subjected to PCR with appropriate primers to amplify DNA fragments containing the SNP, as described by Morales *et al.* (2004). Amplified DNA was digested with the restriction enzyme that discriminated each SNP (Table 1), and the fragments generated were analysed by agarose gel electrophoresis and ethidium bromide staining. To detect the SNP in the *oprL* gene, an artificial restriction site was created in the direct primer, and ‘mismatch PCR’ was performed. In brief, the primer was designed so that its 3' end was 1 bp upstream from the SNP, and it contained a mismatch close to the 3' end, which generated a restriction site for BstUI when the sequence contained a cytosine at the site of the SNP, but not when the base was a thymidine. Therefore, the presence or absence of the BstUI site allowed analysis of the SNP.

Analysis of *fliC*, *exoS* and *exoU*. *P. aeruginosa* strains contain one of two variants of the *fliC* gene (a or b type), which encode flagellins of different molecular mass (Spangenberg *et al.*, 1998), and one or no copies of *exoS* or *exoU*, which encode type III secretion effector proteins. Most strains encode either *exoS* or *exoU* (Feltman *et al.*, 2001). Therefore, the presence of *exoS* and *exoU* and the *fliC* type were established for each strain by PCR.

Sequence analysis at the *muc* locus. One loop of a single *P. aeruginosa* colony, grown on blood agar, was processed for DNA extraction using a commercial DNA isolation kit (Qiagen), according to the instructions of the manufacturer. PCR amplification of the entire *mucA*, *mucB* and *mucD* genes was carried out using *Taq* DNA polymerase (Qiagen). The following primers (MWG-Biotech) were used: *mucA*, 5'-CTC GTG AAG CAA TCG ACA AA-3' and 5'-AAA AGC AAC AGG GAG GTG GT-3'; *mucB*, 5'-ATC CGC CGT CAG TGG TAC AG-3' and 5'-CGA GCA GGA CGA GCA GGT AC-3'; and *mucD*, 5'-GTC CGA TTC GGC CTG AGT CT-3' and 5'-ACG CAG GTA ACG GAT TGA CG-3'. The amplified DNA samples were sequenced by standard automated DNA sequence technology employing the primers described above and the following additional internal primers for *mucB* and *mucD*: *mucB* int1, 5'-CAG TGG TCC TTG CGG TGA CT-3' and *mucB* int2, 5'-TTC AGC AGC AGC GAC TTC AA-3', and *mucD* int1, 5'-GAT CAA CCC GGG TAA CTC CG-3' and *mucD* int2, 5'-AGA TCT GCG AGT TGA TGC CG-3'. The sequence results were compared with the strain PAO1 sequence (www.pseudomonas.com) by the BLAST program at the NCBI database (www.ncbi.nlm.nih.gov/blast/), in order to determine the occurrence of sequence variants within the *muc* gene cluster.

The relatedness of strains by *mucABD* or multilocus genotype was calculated by parsimony analysis with the program PARS of the phylogenetic software package PHYLIP version 3.6 (alpha 3) (Felsenstein, 2002).

Table 1. Multilocus SNP genotyping of *P. aeruginosa*

The sequences of the *P. aeruginosa* strains PA103 (*exoU*), PAK (*fliC* a type) and PAO1 (all other genes) were taken as reference.

Locus	Restriction enzyme	SNP or presence of gene (see Fig. 1)	Designation
<i>oriC</i>	AlwNI	T267C	I
<i>oprL</i>	BstUI	T396C	II
<i>fliC</i> a	MspAII	A672T	III
<i>alkB2</i>	MspAII	G471A	IV
<i>citS</i>	Acil	A533G	V
	MnlI	G896C	VI
<i>oprI</i>	Acil	T195C	VII
<i>ampC</i>	MspI	G789A	VIII
	BstUI	C1060T	IX
	Hinfl	G1150A	X
	AlwNI	G1166C	XI
	AgeI	T1585C	XII
	BsrI	C1639A	XIII
<i>fliC</i>		a type	XIV
<i>fliC</i>		b type	XV
<i>exoS</i>			XVI
<i>exoU</i>			XVII

Alginate determination. For the quantitative determination of alginate production by *P. aeruginosa* patient isolates, the carbazole assay was used (Lam *et al.*, 1980). Briefly, *P. aeruginosa* was first cultured aerobically in trypticase soy broth (Oxoid) overnight at 37 °C, then plated on ‘*Pseudomonas* isolation agar’ containing 1 % glycerol, v/v, as a carbon source, and incubated for 24 h at 37 °C, followed by determination of alginate production. For uronic acid detection, growth from three different plates was pooled. The uronic acid content was normalized by measuring the protein content (Bio-Rad).

RESULTS

Sequence diversity of the *mucABD* locus

Sequencing of the *mucA*, *mucB* and *mucD* genes in 37 sequential *P. aeruginosa* strains from 10 CF patients (Table 2) and in nine environmental isolates revealed 61 SNPs (Table 3) corresponding to 2 % sequence diversity at this locus, which is significantly higher than the mean sequence diversity of 0.5 % in the *P. aeruginosa* genome (Spencer *et al.*, 2003). The overrepresentation of transitions (83 %) and synonymous substitutions (80 %) is similar to that found for evolutionarily neutral SNPs in other loci (Kiewitz & Tümmeler, 2000) or in the bulk genome (Spencer *et al.*, 2003). The 61 SNPs segregated into 18 *mucABD* genotypes, one of which split into two variants (Table 2, Figs 1 and 2). The largest divergence of the *mucABD* sequence was observed between the completely sequenced reference strains PAO1 and PA14, the latter represented in our panel by one environmental and five CF isolates (Fig. 1).

Of the 14 non-synonymous nucleotide substitutions, the two common valine–isoleucine polymorphisms I137V and

V441I and the sequence variant Q225E in *mucD* were found in more than one SNP genotype. All other amino acid substitutions were specific for a single SNP genotype, including the three non-conservative substitutions S113F in *mucB* and W171R and S363N in *mucD*. A representation of Dayhoff’s mutation odds matrix (Dayhoff *et al.*, 1978) by projecting the matrix using multidimensional scaling (French & Robson, 1983; Taylor, 1986) indicated that these three amino acid replacements at least were not neutral; however, it is not yet known whether any substitution modulates the function of the gene product.

Comparison of the *mucABD* single locus genotype with the genome-wide SNP and Spel fragment genotype: evidence for intragenic mosaicism and interclonal recombination

The sequential isolates from individual patients belonged either to one clone (three patients) or to two clones (seven patients) (Table 2). Four clones (A, C, D and L; Table 2) were detected in more than one patient. Environmental and CF isolates of the abundant clones C (Dinesh *et al.*, 2003) and D (strain PA14; Liberati *et al.*, 2006) were identical in their *mucABD* sequences (Fig. 1).

The classification by *mucABD* genotype corresponded for most strains with that by genome-wide multilocus SNP and anonymous macrorestriction fragment genotypes (Table 2, Fig. 1). Differences were taken as evidence for intra- or interclonal exchange of sequence at the *muc* locus. First, unrelated clones could share the same *mucABD* sequence. One set was represented by clones I and L, the other set by

Table 2. *P. aeruginosa* sequential isolates from CF patients

Strain types: the genome-wide multilocus SNP genotype is indicated by upper-case type, the *mucABD* genotype by lower-case type, and the *SpeI* genomic fragment pattern genotype by upper-case Greek type. Strains of PFGE genotype Γ belong to the abundant clone C (Dinesh *et al.*, 2003), and strains of genotype Δ belong to clone PA14 (Liberati *et al.*, 2006).

Patient	Age at onset of colonization (years)	Strain	Colonization time (years)	Alginate [μg uronic acid (μg protein) $^{-1}$]*	Multilocus SNP genotype	<i>mucABD</i> genotype	<i>mucA</i> stop mutation	PFGE genotype
Patient 1	7.4	RP1	0	0.02	A	a	No	A
		RP2	0	0.02	A	a	No	A
		RP73	17.5	0.01	B	b	No	B
		RP74	17.5	0.01	B	b	No	B
Patient 2	12.5	SG1	0	0.01	A	a	No	A
		SG2	2.1	0.06	C	c	ΔG430	Γ
		SG57	15.8	0.01	A	a	No	A
		SG58	15.8	0.01	A	a	No	A
Patient 3	10.6	NN1	0	0.01	C	c	No	Γ
		NN2	0	0.01	C	c	No	Γ
		NN84	16.3	0.03	D	d	C352T	$\Delta 1$
Patient 4	13.8	BT1	0	0.03	D	d	T412C C505T	$\Delta 2$
		BT2	0	12.1	D	d	C505T	$\Delta 2$
		BT72	15.8	1.2	D	d	C505T	$\Delta 2$
		BT73	16.3	6.1	D	d	C505T	$\Delta 2$
Patient 5	19.5	KB1	0	0.01	E	e1	No	E
		KB2	0.9	0.02	E	e2	No	E
		KB66	15.8	0.02	F	f	G474A ΔC484	Z
Patient 6	3.9	AA1	0	0.01	G	g	No	H
		AA2	0.5	0.03	H	h	No	Θ
		AA43	7.5	1.0	H	h	ΔA358	Θ
		AA44	7.5	0.04	H	h	ΔG430	Θ
Patient 7	7.5	TR1	0	0.01	I	i	ΔG430	I
		TR2	0	0.01	I	i	ΔG430	I
		TR66	12.8	0.02	I	i	No	I
		TR67	13.5	3.9	I	i	ΔG430	I
Patient 8	13.2	MF1	0	0.01	J	j	No	K
		MF2	0	0.01	J	j	No	K
		MF51	10.1	0.03	J	j	No	K
		MF52	10.1	0.01	K	k	No	Λ
Patient 9	17.0	KK1	0	0.01	L	i	No	M1
		KK2	0	0.01	L	i	ΔGT400 ΔG430	M1
		KK71	12.6	0.01	L	i	No	M1
		KK72	12.6	0.01	L	i	No	M1
Patient 10	8.1	BST1	0	0.02	M	m	No	N
		BST2	1.4	0.02	L	i	No	M2
		BST44	4.5	0.01	L	i	ΔG430	M2

*The alginate contents of the PAO1 strain and of its isogenic PDO300 mutant (Mathee *et al.*, 1999) harbouring the *mucA* stop mutation ΔG430 were 0.01 and 2.7 μg uronic acid (μg protein) $^{-1}$, respectively.

clone H and the environmental isolate PT6. In other words, the same *mucABD* genotype was observed in different genomic backgrounds. Second, the clones B and H represented by strains RP73 and RP74, and AA2, AA43, AA44 and PT6, respectively, shared an identical SNP pattern in *mucA*, *mucB* and the 5' portion of *mucD*, but differed in their SNP pattern 3' of SNP32 (Fig. 1), indicating that these two *mucABD* genotypes emerged by intragenic recombination.

Evidence for intragenic recombination at the *muc* locus was derived not only from interclonal, but also from intracolon sequence comparison. The first case was the strains KB1 and KB2 which were retrieved from the same patient's lungs within the first year of colonization with *P. aeruginosa*. The two isolates were perfectly matched in their genome-wide SNP and *SpeI* fragment genotypes, but differed in their *mucA* SNP genotype, with the *mucBD* genotypes being identical (Fig. 1). Since it is extremely unlikely that three

Table 3. SNPs in the *mucABD* locus

SNP no.*	Sequence variant†	Frequency‡
<i>mucA</i>		
1	T126C, s	8 (17)
2	T133C, s	10 (21)
3	C156T, s	7 (15)
4	C188G, A-G	1 (2)
5	T198G, s	8 (17)
6	G237C, E-D	1 (2)
7	A243G, s	7 (15)
8	G267A, s	1 (2)
9	A342G, s	47 (98)
10	A358T, T-S	1 (2)
11	C360A, s	1 (2)
12	T381G, s	7 (15)
<i>mucB</i>		
13	T90C, s	1 (2)
14	T195C, s	8 (17)
15	C197T, A-V	3 (6)
16	C255T, s	7 (15)
17	C336T, s	8 (17)
18	C338T, S-F	10 (21)
19	G342A, s	9 (19)
20	C390T, A-V	1 (2)
21	T411C, s	8 (17)
22	T420C, s	8 (17)
23	G486A, s	10 (21)
24	G586T, A-S	6 (13)
25	A631G, T-A	8 (17)
26	A651G, s	8 (17)
<i>mucD</i>		
27	G183T, s	10 (21)
28	G231A, s	9 (19)
29	G252A, s	1 (2)
30	G267C, s	46 (96)
31	T294C, s	7 (15)
32	C345T, s	1 (2)
33	T406C, s	18 (38)
34	A409G, I-V	18 (38)
35	G417A, s	5 (10)
36	T511C, W-R	2 (4)
37	T582G/C, s/s	32 (67)/6 (13)
38	T645C, s	8 (17)
39	T657C, s	1 (2)
40	C673G, Q-E	7 (15)
41	C732T, s	3 (6)
42	T747C, s	8 (17)
43	C774T, s	1 (2)
44	T804C, s	13 (27)
45	C819T, s	12 (25)
46	G876A, s	2 (4)
47	A897G, s	13 (27)
48	C912T, s	13 (27)
49	T927C, s	15 (31)
50	G1017A, s	4 (8)
51	T1047C, s	5 (10)

Table 3. cont.

SNP no.*	Sequence variant†	Frequency‡
52	C1069T, s	9 (19)
53	C1088A, S-N	5 (10)
54	G1135A, A-T	1 (2)
55	C1170T, s	1 (2)
56	C1290T, s	2 (4)
57	G1302A, s	2 (4)
58	G1321A, V-I	9 (19)
59	G1377A, s	9 (19)
60	A1389G, s	9 (19)
61	T1404C, s	9 (19)

*SNP no. used in Fig. 1.

†The sequence of the *P. aeruginosa* PAO1 strain was taken as a reference.

‡The frequency of strains carrying the non-PAO variant is given as an absolute number and as a percentage of the investigated strain panel (in parentheses). The frequencies are only rough estimates of the frequency of the SNPs in the *P. aeruginosa* population, because in numerous cases sequential isolates of the same clone were sequenced (see Fig. 1, Table 2).

successive mutations at common SNP positions occurred *de novo*, one strain must have incorporated its *mucA* gene from another *P. aeruginosa* clone. The second case was the clone G strains AA1 and PT20 which had been isolated in 1985 and 1992, at a distance of 300 km from one another, from CF airways and a sanitary facility, respectively. The two strains were identical in their genomic SNP genotype and most *muc* sequences, but differed in the outermost SNPs at the 5' end of *mucA* and the 3' end of *mucD*. The genomically related mushroom isolate ATCC 33818 shared the *mucA* sequence with strain PT20 and the *mucD* sequence with strain AA1 (Fig. 1). Hence, two recombinations in the *muc* locus can explain the emergence of three related mosaic *muc* genotypes.

***mucA* stop mutations in CF isolates: frequent recurrent mutations**

Unequivocal loss-of-function stop mutations were identified in the *mucA* gene of 14 of our 37 CF isolates (38%), but in none of the nine environmental isolates that were sequenced in the *mucABD* genes. No loss-of-function mutations were detected in *mucB* or *mucD*. *mucA* stop mutations were associated with five *mucABD* genotypes (Fig. 2), and were observed with similar frequency in strains recovered from both healthier and more severely ill patients (Table 2) (chi-square test: $P=1$). Two C-to-T transitions, numerous single nucleotide deletions and two double mutations were observed (Table 2). Four of the six different mutations were located in stretches of thermodynamically stable double strands with >80% GC content.

Fig. 1. Multilocus genome-wide SNP genotypes (roman numerals; see Table 1) and *mucABD* single-locus SNP genotypes (arabic numerals; see Table 3) of 37 sequential *P. aeruginosa* isolates from 10 individuals with CF, and nine environmental isolates. The sequenced *P. aeruginosa* strain PAO1 was taken as a reference. Environmental strains are designated by italic type and an asterisk.

mucA mutations have been reported to arise in *P. aeruginosa* during the stage of chronic colonization, when the strains become mucoid (Spencer *et al.*, 2003). However, in our cohort, initial isolates from the first *P. aeruginosa*-positive sputum from patients 4, 7 and 9 carried *mucA* mutations (Table 2). These three patients became positive for *P. aeruginosa* after discharge from a stay at a CF ward, or after return from a CF rehabilitation centre, indicating that their *mucA* mutations were acquired with the initially colonizing strain from an external CF-related source. This suspicion of nosocomial acquisition is supported by the phenotypes of the initial isolates that are typically observed in samples retrieved from chronically colonized CF lungs. The strains from these three patients were mucoid and/or resistant to antipseudomonal agents.

In patients 2, 3, 5, 6 and 10, *mucA* stop mutations were detected in just one isolate per patient, which strongly

suggests that the mutations had emerged *de novo* in the CF lung habitat. Half of all the mutations consisted of a deletion of a G in a string of five G residues located at positions 426–430 in a GC-rich stretch of the *mucA* gene. This Δ G430 deletion occurred in four *mucABD* haplotypes (Table 2, Fig. 2), which is proof of recurrent mutations on diverse genetic backgrounds.

Recent sequence analyses of the *mucA* gene in large collections of CF isolates from North America (Spencer *et al.*, 2003; Yoon *et al.*, 2006), Australia (Anthony *et al.*, 2002) and Europe (this study) have identified insertions or deletions in 18 strains, and 21 different stop mutations in 92 strains. More than 80 % of the frameshift or nonsense mutations that give rise to a premature stop signal were clustered within the 100 bp stretch from nt 340–439 of the 585 bp *mucA* gene (Fig. 3). The most frequent Δ G430 mutation accounted for 40 % of all stop mutations. The

Fig. 2. Dendrogram based on *mucABD* genotypes of 37 CF isolates and nine environmental isolates, with *P. aeruginosa* PAO1 and PA14 as reference strains. Strains carrying a *mucA* stop mutation are designated by bold type. Environmental strains are designated by an asterisk. Bar, 1 SNP.

recurrent emergence of the Δ G430 mutation in independent CF lung habitats and unrelated *mucABD* genetic backgrounds (Table 2, Fig. 2) is in agreement with *in vitro* studies showing that this homopolymeric tract is prone to single nucleotide deletion upon exposure of *P. aeruginosa* biofilms to reactive oxygen species, a scenario envisaged to occur in the CF lung (Mathee *et al.*, 1999).

Stop mutations in *mucA* are known to lead to alginate overproduction and conversion to a stable mucoid phenotype in *P. aeruginosa* (Martin *et al.*, 1993b). We were able to confirm the causative role of the loss-of-function mutation in *mucA* for mucoidy in the reference strain PAO1 and its isogenic single mutant Δ G430 *mucA* (footnote in Table 2). This association, however, was not so evident for the CF isolates. Just five of the 14 *mucA* stop mutants were overexpressing alginate (Table 2). During the chronic colonization of CF airways, *P. aeruginosa* is continuously accumulating mutations in its genome

Fig. 3. Frequency and localization of stop mutations of the *mucA* gene of *P. aeruginosa* strains collected from the airways of individuals with CF in North America (Spencer *et al.*, 2003; Yoon *et al.*, 2006), Australia (Anthony *et al.*, 2002) and Europe (this study).

(Smith *et al.*, 2006); therefore, secondary site mutations probably caused the reversion to a non-mucoid morphotype in the majority of *mucA* mutant strains.

DISCUSSION

Chromosomal mosaic genes are a major source of genetic diversity, and are common in naturally transformable bacteria such as neisseriae and streptococci (Hakenbeck, 1998). In the non-transformable *P. aeruginosa*, mosaicism is known in the pyoverdine biosynthesis locus (Smith *et al.*, 2006), *ampC* (PA4110) (Spangenberg *et al.*, 1998), *fleP* (PA1096) (Arora *et al.*, 2001), *fliC* (PA1092) (Spangenberg *et al.*, 1998) and *oprD* (PA0958) (Pirnay *et al.*, 2002b). This report adds the *muc* operon to the list of mosaic genes. Compared to the number of analysed clones and isolates, the frequency of intragenic recombination may appear to be high; however, this does not imply that this locus is subject to evolutionary forces additional to those of the bulk of the genome. Bacteria such as the ubiquitous and metabolically versatile *P. aeruginosa* are believed to have very large population sizes (Lynch & Conery, 2003), and correspondingly are expected to evolve under purifying or negative natural selection, i.e. natural selection acting to decrease the frequency of deleterious alleles. The quantitative criterion is a low ratio of non-synonymous (d_N) over synonymous (d_S) nucleotide substitutions, and indeed, low d_N/d_S ratios of 0.14 are observed when large datasets of phylogenetically independent pairs of genes are compared between closely related pairs of bacterial species (Friedman *et al.*, 2004; Jordan *et al.*, 2002). A significant excess of d_N over d_S nucleotide substitutions is interpreted as evidence for the action of positive selection. The d_N/d_S ratio of 0.27 for the *muc* locus, however, is not significantly different from the global d_N/d_S ratio of $\sim$ 0.16 for the *P. aeruginosa* core genome (Spencer *et al.*, 2003). Hence, we conclude that the *muc* locus, like the core genome, is subject to ongoing purifying selection.

Although at first glance the observed intragenic recombination rate seems to be high, it is in complete accordance with earlier reports that recombination is frequent in *P. aeruginosa*, and that correspondingly the population has an epidemic non-clonal population structure (Kiewitz & Tümmler, 2000; Pirnay *et al.*, 2002a). All genotypes, each of which is characterized by non-random association of alleles, are in linkage equilibrium to each other. In other words, the frequency of recombination is high enough to prevent linkage disequilibrium among genes and hitchhiking effects on the phenotype. Previous publications on the population genetics of *P. aeruginosa* have focused on the diversity of unrelated clones. By sequencing several strains of the same clone, we now demonstrate that interclonal recombination contributes to intraclonal variation and the generation of mosaic genes. The colonized CF lung is characterized by high numbers of *P. aeruginosa* and only a few other bacterial competitors (Breitenstein *et al.*, 1997). Such a scenario may facilitate the exchange of homologous genes between two or more *P. aeruginosa* clones.

Sequential isolates from CF lungs have been extensively characterized in one index case who became chronically colonized with *P. aeruginosa* very early, by the age of 6 months (Smith *et al.*, 2006). Complete genome sequencing of clonally related month 6 and month 96 isolates uncovered the fixation of 68 mutations in the chromosome of the late isolate. No stop mutation in the *mucA* gene was observed in the month 96 isolate. A previous analysis on 36 sequential isolates recovered from the same patient during the first 30 months of airway colonization, however, identified eight mucoid strains, six of which carried either the $\Delta G357$ or the $\Delta G430$ stop mutation in *mucA* (Spencer *et al.*, 2003). Lineages harbouring *mucA* mutations have not been detected in month 60 and month 96 isolates (see Fig. 1 in Smith *et al.*, 2006). The disappearance of *mucA* mutants from the lungs of this early colonized patient is in accordance with our observation in the older patients 2 and 9 of our cohort (Table 2), who also lost *mucA* mutants during colonization. Our study on initial and late isolates from 10 patients moreover demonstrates that loss-of-function mutations in *mucA* either emerge *de novo* in the CF lung (see Table 2, patients 2, 3, 5, 6 and 10) or are acquired by nosocomial transmission (patients 4, 7 and 9).

In summary, the spectrum of loss-of-function mutations in the *mucA* gene of CF isolates is dominated by the major mutation $\Delta G430$ and a cluster of further stop mutations within 100 bp upstream of $\Delta G430$. The dominant mutations are recurrent mutations on different *mucABD* haplotypes. No mutations were detected in the negative regulator *mucB* or in *mucD*, which plays a dual role in the regulation of alginate production, as well as in temperature resistance (Wood & Ohman, 2006). Sequencing of sequential isolates moreover provided direct experimental evidence for intragenic recombination as a means of generating intraclonal diversity in *P. aeruginosa*.

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