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Hypermutability in environmental *Pseudomonas aeruginosa* and in populations causing pulmonary infection in individuals with cystic fibrosis

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Pseudomonas aeruginosa is the pathogen most commonly associated with morbidity and mortality in cystic fibrosis (CF) patients. The host–pathogen interactions responsible for progressive CF lung diseases are complex. However, there is growing interest in the role of hypermutable *P. aeruginosa* (that is, those strains with an increased mutation frequency due to mutations in mismatch repair and error prevention genes), in terms of both bacterial adaptation and antimicrobial resistance. The prevalence of hypermutable *P. aeruginosa* in chronic CF infection has been established, and at 37 % is surprisingly high. To the authors' knowledge, there are no reports of prevalence during the early stages of infection, in environmental pseudomonas, which are believed to be the primary source of infection, and in epidemic strains, which have emerged as a major challenge. The aim of this study was to establish the prevalence of hypermutable *P. aeruginosa* in these pseudomonas populations. The hypothesis was that hypermutability would be rare in early and in environmental *P. aeruginosa* but in contrast would explain the relatively recent emergence of epidemic strains. It was found that 10/100 (10 %) of early isolates were strong or weak mutators, suggesting that the CF lung is not the only factor influencing the existence of mutators in this group of patients. Two weak mutators (6 %) were found in 32 environmental isolates. Only two of 15 (13 %) epidemic *P. aeruginosa* strains were hypermutable, and although closer analysis revealed this issue to be complex, on the whole the data suggested that the atypical characteristics of these highly transmissible strains cannot solely be explained by this phenomenon. The higher than predicted prevalence of mutators in early infection, and in environmental isolates, reinforces the importance of early and aggressive treatment for *P. aeruginosa* infection in CF.

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous, highly adaptable Gram-negative bacillus and an opportunistic pathogen causing a variety of infections in humans and animals (Goldberg, 2000). Individuals with cystic fibrosis (CF) are particularly susceptible to lung infection by a limited spectrum of microbial pathogens, of which *P. aeruginosa* is the most important cause of morbidity and mortality. Initial *P. aeruginosa* infection in CF is believed to derive from environmental sources and occurs early, in the infant or young child (Gibson *et al.*, 2003). The non-mucoid colonial form of *P. aeruginosa* typical of early infection is relatively susceptible to antibiotics and can be eradicated if treated rapidly and aggressively (Burns *et al.*, 2001). However, if treatment is delayed or ineffective, a chronic mucoid phenotype eventually emerges. The establishment of chronic infection with mucoid *P. aeruginosa* is a critical development, since pseudomonas alginate in association with bronchial mucin forms complex biofilms that in turn lead to antibiotic resistance, frustrated phagocytosis and a vicious cycle of neutrophil-mediated inflammation.

In the last decade, the emergence of highly transmissible strains of *P. aeruginosa* has presented yet another challenge to the CF community. There have been several well-documented outbreaks caused by 'epidemic' strains, which are typically multi-drug resistant (Cheng *et al.*, 1996;

Abbreviation: CF, cystic fibrosis.

McCallum *et al.*, 2001; Armstrong *et al.*, 2002). Some of these strains have unusual characteristics such as atypical colonial morphology and an enhanced ability to survive aerosolization and to infect extrapulmonary sites (Jones *et al.*, 2003; Taylor *et al.*, 1992; Panagea *et al.*, 2005; Salunkhe *et al.*, 2005; Chambers *et al.*, 2005). The microbial factors responsible for enhanced transmissibility are unclear. However, it is clear that these strains pose a serious problem for cross-infection control and antimicrobial therapy.

The molecular mechanisms underlying the adaptation of *P. aeruginosa* to the CF lung are poorly understood, but undoubtedly complex. A recent longitudinal study by Smith *et al.* (2006) demonstrated the importance of mutation in the adaptation of *P. aeruginosa* to the CF lung, and the role that a hypermutation phenotype might play in bacterial pathogenesis in general. Hypermutable bacteria, or 'hypermutators', are bacteria that have an increased (up to 1000-fold) mutation rate. This phenomenon was initially identified in pathogenic *Escherichia coli* (Herman & Modrich, 1981), and occurs when defects in the bacterial DNA repair or error avoidance genes lead to a general increase in mutation frequency. Some of these secondary mutations may have a positive effect on bacterial survival, including advantageous properties such as antibiotic resistance and increased virulence. Recently, there has been much interest in the role of hypermutable bacteria in CF lung disease. A seminal study by Oliver *et al.* (2000) revealed that 37 % of CF patients chronically infected with *P. aeruginosa* were colonized by hypermutable strains; this contrasted with the absence of hypermutators in acute pseudomonas infections of non-CF patients. It has been suggested that the unique environment of the CF lung, with its variable nutrient supply, fluctuating inflammatory response and exposure to aggressive and prolonged antibiotic therapy, is highly susceptible to the establishment of a stable hypermutable phenotype (Oliver *et al.*, 2000; Ciofu *et al.*, 2005). Subsequently, other studies have shown that the mutator phenotype is relatively common in chronic *P. aeruginosa* infection in other lung disorders exhibiting a similar pathology to CF (Maciá *et al.*, 2005). Hypermutable populations have also been identified in other major CF pathogens, namely *Haemophilus influenzae* and *Staphylococcus aureus* (Watson *et al.*, 2004; Román *et al.*, 2004; Prunier *et al.*, 2003).

The association between antimicrobial resistance and hypermutability in chronic CF infections is well documented (Oliver *et al.*, 2000, 2004; Maciá *et al.*, 2004, 2005) and has important implications for antimicrobial therapy, in particular early treatment on first isolation of *P. aeruginosa* infection to prevent or delay chronic infection (Burns *et al.*, 2001; Maciá *et al.*, 2005). It is also clear that hypermutable *P. aeruginosa* have a propensity for survival in the chronically infected CF lung (Oliver *et al.*, 2000). However, to our knowledge, the mutator phenotype has not been studied in other *P. aeruginosa* populations involved in CF lung disease. Thus in the present study, we assessed the prevalence of hypermutators within three different *P. aeruginosa* populations: (1) early clinical isolates from the CF lung, (2) environmental isolates that are considered to be the source of the majority of CF populations and (3) epidemic strains exhibiting multiple drug resistance and enhanced transmissibility. In *P. aeruginosa* the most commonly affected genes leading to hypermutabilty are those belonging to the mismatch repair system, in particular *mutS* (Oliver *et al.*, 2000, 2002). Isolates found to be strongly hypermutable were investigated further to define the genotypic basis of their hypermutator phenotype.

METHODS

Strains. Bacterial isolates were confirmed as *P. aeruginosa* using the API 20NE system and PCR (Spilker *et al.*, 2004). Clonality was excluded across all three groups by PFGE (Butler *et al.*, 1995), with the exception of three patients whose primary infection involved an epidemic strain, and two of the environmental isolates. *P. aeruginosa* PAO1 and the hypermutable strain, *P. aeruginosa* J3295 (RH04 00 0003-2) were used as negative and positive controls, respectively. One hundred early isolates from 95 patients originated from respiratory samples from nine CF clinics in the UK and one clinic in Belgium; these were held in the Edinburgh CF Microbiology Laboratory and Strain Repository (ECFML) and had a non-mucoid phenotype, consistent with initial colonization. Isolates with a mucoid phenotype were excluded as this suggested established infection rather than early colonization. The panel of 15 epidemic strains comprised isolates from 13 separate outbreaks in CF clinics in the UK, Australia, Germany and the Republic of Ireland (Table 1). Twenty-three environmental strains were cultured from water, soil, or vegetable matter following enrichment in acetamide broth (Kelly *et al.*, 1983; Curran *et al.*, 2005) and overnight growth on *Pseudomonas* Isolation Agar (BD Biosciences) at 37 °C. Nine environmental isolates were kindly provided by Professor Peter Vandamme (Universiteit Gent, Belgium).

Phenotypic determination of mutation frequency. Phenotypic mutation frequencies were determined by resistance to rifampicin, based on the method described by Oliver *et al.* (2000). Briefly, independent triplicate 20 ml Mueller–Hinton broth (MHB) cultures of each strain (3–5 colonies) were grown overnight in an orbital incubator at 37 °C. Cells were pelleted, resuspended in 1 ml MHB and serial 10-fold dilutions were prepared in sterile saline. Samples (100 µl) of the neat, 10^{-1} and 10^{-2} dilutions were plated on Mueller–Hinton agar (MHA) with 300 µg rifampicin ml^{-1}, and 100 µl of the 10^{-6}, 10^{-7} and 10^{-8} dilutions were plated on to MHA without antibiotics. After 36 h incubation, colonies were counted and 10 colonies of each morphotype were streaked on to new antibiotic plates to assess mutant stability. The mean number of mutants was calculated for each strain. For any strains displaying mutation jackpot (that is, the chance appearance of a large number of mutants in one of the triplicate experiments), the triplicate experiment was repeated. Strains were divided into four categories based on mutation frequency. These were: normomutable (based on a mutation frequency, *f*, close to the modal point of the distribution of mutation frequencies, in this case between 7×10^{-9} and 2×10^{-7}), weak mutators (strains with a mutation frequency of $2 \times 10^{-7} > f < 1 \times 10^{-6}$) and strong mutators ($f \geqslant 1 \times 10^{-6}$; approximately 20-fold greater than that of PAO1). Finally strains with $f < 7 \times 10^{-9}$ were considered to be hypomutable. To monitor reproducibility, PAO1 and a known hypermutator, strain J3295 (RH04 00 0003-2), were tested in triplicate in each experiment.

Table 1. Epidemic strains screened for hypermutability in this study

Strain no.	Epidemic	Reference
H129	LES	Cheng *et al.* (1996)
C3425	MAN	Jones *et al.* (2001)
C4448	Clone C Germany, P10118	Römling *et al.* (1994)
C4450	Midlands, P9245	Scott & Pitt (2004)
C4269, C4270	Brisbane, Australia	Armstrong *et al.* (2003)
C3796, C3798	Melbourne, Australia	Armstrong *et al.* (2002)
E859	Sheffield	Edenborough *et al.* (2004)
E1632	Yorkhill, Glasgow	Unpublished
E1476	Dublin 1	Unpublished
E1453	Dublin 2	Unpublished
E1457	Dublin 3	Unpublished
E1609	Dublin 4	Unpublished
E1284	Bristol	Unpublished

PCR and sequencing. Ten primer pairs were designed to span the 2.5 kb *mutS* gene, based on the sequence from the PAO1 genome sequence database (http://www.tigr.org/) (Table 2). For DNA extraction, a boiled preparation was made for each strain using the following method. One or two colonies were taken from overnight growth on nutrient agar (Columbia agar, Oxoid) and mixed with 20 µl lysis buffer (0.25 %, w/v, SDS in distilled water, 0.05 M NaOH, 92.8 ml MilliQ water). After incubation for 15 min at 95 °C, the sample was pulsed in a centrifuge, 180 µl MilliQ water was added and a final centrifugation was performed for 5 min at 16 110 ***g***. Each 25 µl PCR reaction contained the following reagents: approximately 20 ng DNA, 250 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$ (Qiagen), 1× PCR buffer (Qiagen), 20 pmol of each primer (Invitrogen), 1.25 U Hot star*Taq* polymerase (Qiagen) and sterile distilled water. DNA was amplified in a TC-312 thermal cycler (Techne). PCR conditions were as follows: 1 cycle of 95 °C for 15 min, followed by 30 cycles of 95 °C for 1 min, 1 min at the appropriate annealing temperature for the *mutS* primer pair used (Table 2) and 72 °C for 1 min, with a final extension step of 72 °C for 10 min. Samples (10 µl) of PCR product were run alongside a 1 kb Plus DNA ladder (Invitrogen) on a 1.2 % pre-cast E-gel (Invitrogen). PAO1 was used as a positive control and the negative control consisted of PCR reaction mix without DNA. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced using both forward and reverse primers. Sequencing conditions were as follows: 1 cycle of 95 °C for 1 min, 25 cycles of 95 °C for 30 s, 45 °C for 15 s, and 60 °C for 4 min. The sequencing mixture consisted of 4 µl ABI PRISM BigDye terminator v3.1 cycle sequencing mix (Applied Biosystems), 40 pmol primer (the same primers as had been used for PCR) and 1–2 µl DNA, depending on the concentration of DNA. The mixture was made up to 20 µl with sterile distilled water. Vector NTI 8 suite (InforMax) was used to analyse DNA sequences. Sequences were compared to the published PAO1 genome sequence (http://www.tigr.org/).

Table 2. PCR primer sequences and annealing temperatures for *P. aeruginosa* mismatch repair and error prevention genes

Primer	Primer sequence*	Annealing temp. (°C)
mutS1	5′-ATGACCGACCTCTCCCAGCA-3′	62
mutS2	5′-TCTGGAATCAGCAGCTCGGC-3′	
mutS3	5′-GCCGCTTCAGCGTCCAGGAGATAA-3′	65
mutS4	5′-TTTTCGCTCAGCGCCTGCAGCT-3′	
mutS5	5′-GAACTGCTGGCCAAGGCGAT-3′	59
mutS6	5′-TTCGCCAGCACGTCCAGTTC-3′	
mutS7	5′-CCGCATCCATGGCTACTTCA-3′	58
mutS8	5′-AAGATGCGGTCCACCAGGGA-3′	
mutS9	5′-GCGGTTCGTCGAACACACCT-3′	59
mutS10	5′-TAGTTCTCTCCTCAGGCGGG-3′	
mutL1	5′-TCCGCGCAGGAAGAGCTTGT-3′	59
mutL2	5′-ACCGCGTATCCAGCTGCTGA-3′	
uvrD1	5′-TGAACGACGACCTCTCCCTC-3′	59
uvrD2	5′-CTACAGGGCTTCCAGCTTGG-3′	
mutY1	5′-ATGACACCTGAAGGCTTCAA-3′	55
mutY2	5′-ACTGAAGGTATGGGTCAGGC-3′	
mutM1	5′-ATGCCCGAACTACCCGAAGTCG-3′	63
mutM2	5′-TTGGCAGCGCGGGCAGTACA-3′	
mutD1	5′-TGCGTAGCGTCGTACTGGATAC-3′	61
mutD2	5′-TCCAGTTGCGCCCAGAGTGA-3′	
mutT1	5′-CTGGACCAGGAGGAAACGCT-3′	62
mutT2	5′-GTGATTCGTGGCTCCGATGG-3′	

*Primers designed using the PAO1 genome sequence (http://www.tigr.org/).

Hypermutable strains for which no *mutS* mutation was found by sequencing underwent PCR amplification of the other mismatch repair and error prevention genes (*mutL*, *uvrD*, *mutY*, *mutM*, *mutD* and *mutT*) to establish whether any significant deletions in these genes might be responsible for their elevated mutation frequency. PCR conditions were the same as for *mutS*. Annealing temperatures for primers, which were designed using the PAO1 genome sequence database (http://www.tigr.org/), are shown in Table 2.

RESULTS

Phenotypic determination of mutation frequency

Fig. 1(a) shows the distribution of mutation frequencies for the 100 early or first isolates from CF infants and children. Resulting mutation frequencies revealed that 85/100 (85 %) were normomutable; 5/100 (5 %) were hypomutable, 5/100 (5 %) were weak mutators and 5/100 (5 %) were strong

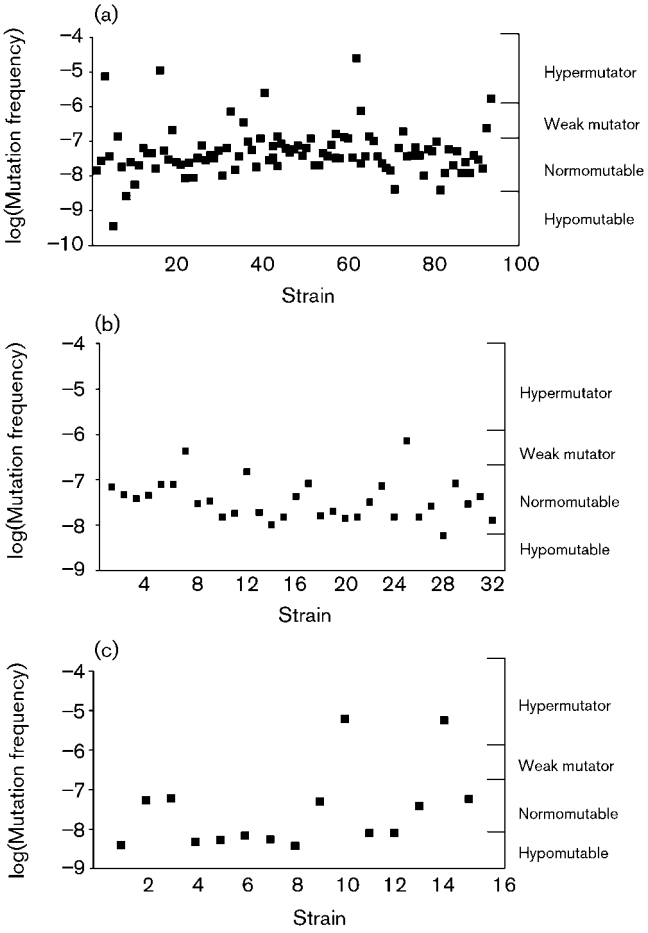

Fig. 1. Rifampicin mutation frequencies for three populations of *Pseudomonas aeruginosa* isolates. (a) 100 early isolates from 95 CF patients; (b) 32 environmental isolates and (c) 15 epidemic strains from CF patients. Isolates were split into four categories based on their mutation frequency: normomutable (those with a mutation frequency of between 7×10^{-9} and 2×10^{-7}), weak mutators (with a mutation frequency $>2\times10^{-7}$ and $<1\times10^{-6}$), strong mutators ($\geqslant1\times10^{-6}$) and hypomutable ($<7\times10^{-9}$). These categories are shown to the right of the figure.

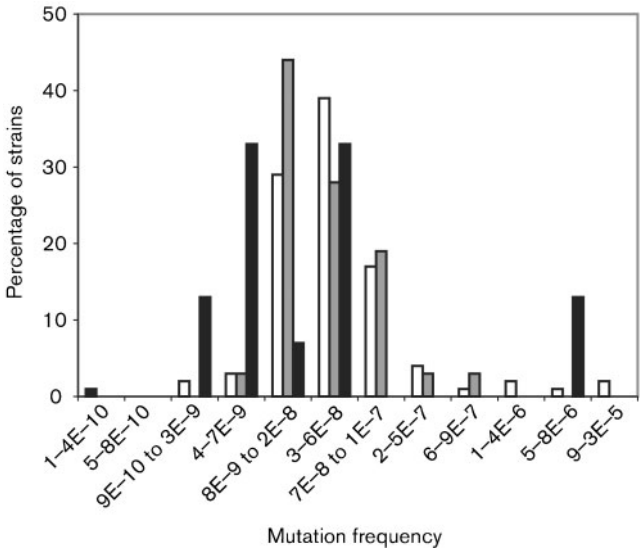

Fig. 2. Distribution of rifampicin resistance mutation frequencies for three *Pseudomonas aeruginosa* populations: 100 early/first isolates from 95 CF patients (white bars), 32 environmental strains (grey bars) and 15 epidemic strains (black bars).

hypermutators (Figs 1a and 2; Table 3). Of the 32 environmental isolates, 1/32 (3 %) was hypomutable and 29/32 (91 %) were normomutable. The remaining 2/32 (6 %) were weak mutators with mutation frequencies of 7.2×10^{-7} and 4.3×10^{-7}, respectively (Figs 1b and 2). Clonality of these two isolates was excluded using PFGE. Of the 15 epidemic strains (Table 1), 7/15 (47 %) were hypomutable, 6/15 (40 %) were normomutable and 2/15 (13 %) were strongly hypermutable (Figs 1c and 2; Table 3). In view of the clinical importance of these epidemic strains, it was decided they should be looked at in greater depth in this study. Five strains from individual patients infected with the Manchester, Liverpool, Bristol and Dublin group 3 epidemic strains (Table 1) were screened with rifampicin. The results revealed some variation in the mutation frequencies between the same epidemic strain from different patients. Intriguingly, two of the three isolates whose hypermutator status differed from those in the cluster were mucoid variants, suggesting this may be important (Table 4). The Bristol epidemic strain, for example, was hypermutable in four out of five patients, but normomutable in the fifth (the mucoid isolate). Interestingly, one of the Manchester isolates had an unusually high mutation rate (3.5×10^{-2}) with respect to the other isolates in this clonal group.

mutS sequencing

mutS sequencing of the five early isolates that were strong hypermutators showed that only two had non-synonymous mutations that might be responsible for their raised mutation frequencies (Table 3). Strain C4649 had a 4 bp insertion after the threonine at amino acid 398, leading to a frameshift, and thus altering the structure of the MutS protein. Strain E2889 had a single point mutation leading to a change in amino acid 636 from glycine to aspartic acid. This change is in domain V of the MutS protein, which has the important function of ATPase activity (Obmolova *et al.*, 2000; Oliver *et al.*, 2002).

Of the two epidemic strains found to be hypermutable, *mutS* screening showed that only one of these, strain E1457, had non-synonymous mutations in *mutS*. Two *mutS* mutations (A545T and Z693G) were found in this strain (Table 3). As far as we are aware, these sequence changes have not been reported previously; however, it seems plausible that one or other is responsible for the elevated mutation frequency since both are part of domain

Table 3. Mutation frequencies and non-synonymous *mutS* mutations in strong mutators

Strain	Source	Mutation frequency	Amino acid position	Nucleotide base position	Codon from PAO1 (amino acid)	Codon change in mutator strain
C4649	Belgium, clinical	7.3×10^{-6}	398	1193 (4 bp insertion: GCAC)	ACC (Thr)	His inserted after Thr (ACG, then CAC)
C4662	Belgium, clinical	1.1×10^{-5}	NC	NC	NC	NC
C3182	Edinburgh (UK), clinical	2.5×10^{-6}	NC	NC	NC	NC
C4488	Manchester (UK), clinical	2.5×10^{-5}	NC	NC	NC	NC
E2889	Paisley (UK), clinical	1.7×10^{-6}	636	1907	GGC (Gly)	GAC (Asp)
E1284	Bristol (UK), clinical (epidemic)	6.3×10^{-6}	NC	NC	NC	NC
E1457	Dublin (RoI), clinical (epidemic)	5.7×10^{-6}	545	1633	GCC (Ala)	ACC (Thr)
			693	2078	GAG (Glu)	GGG (Gly)
E2331*	Papworth (clinical/epidemic)	7.2×10^{-7}	187	559	GCG (Ala)	TCC (Ser)
			286	857	AAC (Asn)	AGC (Ser)
			688	2063	CCT (Leu)	CCC (Pro)

NC, No change from wild-type (PAO1) sequence; RoI, Republic of Ireland.
*Strain E2331 (the Liverpool epidemic strain, derived from a patient in the early isolate group in this study) was the only weak mutator that underwent *mutS* sequencing. *mutS* sequencing was performed to determine whether a mutation in this gene might be responsible for the elevated mutation frequency of strain E2331 with respect to one of the original examples of the Liverpool epidemic strain, H129, which was screened as part of the epidemic group during this study.

V of the MutS protein, which is involved in ATPase activity (Obmolova *et al.*, 2000; Oliver *et al.*, 2002).

PCR amplification of additional mismatch repair and error prevention genes

Early isolates C4662, C3182 and C4488, and epidemic strain E1284, had elevated mutation frequencies that could not be explained by *mutS* mutations. These isolates underwent *mutL*, *mutL*, *uvrD*, *mutY*, *mutM*, *mutD* and *mutT* PCR amplification. The resulting PCR products were the same size as those of the positive control, strain PAO1.

DISCUSSION

In recent years, there has been growing interest in the importance of mutation in bacterial adaptation, and particularly the role of the hypermutator phenotype in bacterial pathogenesis. In CF, the role that hypermutable *P. aeruginosa* might play in chronic lung infection and in antimicrobial resistance has been extensively studied (Oliver *et al.*, 2000, 2004; Ciofu *et al.*, 2005; Maciá *et al.*, 2004, 2005). However, it is not clear at what stage of infection the hypermutable phenotype emerges. In addition, the possibility that hypermutability might be responsible for the occurrence of epidemic *P. aeruginosa* has not, to our knowledge, been investigated. To address these issues, this study investigated the prevalence of hypermutators in three *P. aeruginosa* populations. Our hypothesis was that hypermutability would be rare in early and in environmental *P. aeruginosa* but could explain the phenomenon of epidemic strains. The resulting distribution of mutation frequencies revealed that, rather than isolates being broadly categorized as non-mutators and mutators, there were in fact a number of isolates that were weak mutators, and at the other end of the scale some that exhibited a lower than average mutation rate. This distribution led us to categorize isolates as hypomutable, normomutable, hypermutable or weak mutators, as had been done in a recent study by Baquero *et al.* (2004).

It was surprising to find that 10 % of early isolates were either strong or weak mutators, particularly since the emergence of hypermutators in the CF lung is believed to be uniquely associated with chronic infection (that is, with fluctuating inflammatory responses and prolonged antibiotic therapy) (Oliver *et al.*, 2000; Maciá *et al.*, 2005; Ciofu *et al.*, 2005). To our knowledge, the effect of weak mutators in *P. aeruginosa* infection in CF has not been examined. It has been suggested, however, that in high-density populations of *E. coli*, advantageous mutations will tend to appear in weak mutators, and that these may sometimes prevent the fixation of strong mutators (Chao & Cox, 1983; Baquero *et al.*, 2004).

The higher than predicted number of mutators in early infection suggested that the environment of the CF lung might not be the only factor influencing hypermutability in *P. aeruginosa*, and that at least some of these isolates might be hypermutable prior to infection of CF patients. Alternatively, although we restricted our study to non-mucoid *P. aeruginosa*, in some cases infection may already have existed for some time. Because it is widely believed that the source of most *P. aeruginosa* infection in CF is environmental we screened a panel of 32 environmental isolates for hypermutability. Interestingly, 2/32 (6 %) were

Table 4. Mutation frequencies for epidemic *P. aeruginosa* from individual patients

Epidemic/strain no.	Date of isolation	Mutation frequency
Manchester		
C3373	October 2000	3.1×10^{-8}
C3652	December 2000	2.5×10^{-8}
C3719	March 2001	6.0×10^{-8}
E2637*	August 2005	3.5×10^{-2}
E2840	November 2005	4.3×10^{-8}
Liverpool		
H172	January 1996	4.9×10^{-9}
E2174	April 2005	1.2×10^{-8}
E2886	December 2005	6.9×10^{-8}
E3010	January 2006	2.1×10^{-8}
E3017	January 2006	2.6×10^{-8}
Dublin		
E1457	March 2004	1.2×10^{-6}
E1463	March 2004	2.3×10^{-7}
E1469	March 2004	2.5×10^{-6}
E1611†	May 2004	1.1×10^{-8}
E1650	June 2004	2.4×10^{-6}
Bristol		
E960†	March 2003	8.4×10^{-8}
E1049	April 2003	1.1×10^{-5}
E1236	October 2003	7.9×10^{-6}
E1391	January 2004	6.6×10^{-6}
E1392	January 2004	4.3×10^{-6}

*The unusually high mutation frequency of this strain was reproducible.
†These isolates had a mucoid phenotype.

weak mutators, with one of these being a borderline strong mutator. To our knowledge, hypermutators have not been reported previously for environmental *P. aeruginosa*. It is noteworthy that, as might be expected for both environmental and early populations, the percentage of weak mutators is similar, at 5 % and 6 %, respectively. In addition, these results do indeed suggest that, although the CF lung clearly plays an important role in the selection of hypermutable *P. aeruginosa*, the existence of weak mutators amongst environmental organisms should not be underestimated.

The relatively high number of strong and weak mutators in early CF infection, and to a lesser extent amongst environmental isolates, contrasts strikingly with numbers found in other *P. aeruginosa* populations. For example, results from a study by Gutiérrez *et al.* (2004) supported those of Oliver *et al.* (2000), in finding a very low proportion of *P. aeruginosa* hypermutators amongst intensive care patients infected with this organism. It is not clear why there is such a difference in the mutation frequencies between these studies but it seems plausible that the acute cases of *P. aeruginosa*, presumably derived from the nosocomial environment, may be under a different selective pressure than early and environmental isolates.

Results from the current study reinforce the importance of early and aggressive antimicrobial therapy as advocated by others (Burns *et al.*, 2001; Oliver *et al.*, 2004; Maciá *et al.*, 2005). Oliver *et al.* (2004) addressed this problem with regard to chronic *P. aeruginosa* infection. They concluded that combinations of antipseudomonal agents should be used to minimize the selection for hypermutable antibiotic-resistant mutants. A further informative study supports the relevance of combined therapy for *P. aeruginosa* infection. Maciá *et al.* (2006) investigated mice infected with hypermutable *P. aeruginosa* and showed that resistance could be suppressed by appropriate therapy with more than one antibiotic.

Epidemic strains of *P. aeruginosa* are a relatively recent concern for CF patients. There have been several well-documented studies on transmissible strains, revealing in some cases a capacity for increased antimicrobial resistance, increased transmissibility factors and superinfection of existing *P. aeruginosa* infections (Jones *et al.*, 2001, 2003; Panagea *et al.*, 2005; McCallum *et al.*, 2001). It seemed plausible that the characteristics favouring the survival and spread of these atypical transmissible *P. aeruginosa* isolates could be explained by the phenomenon of hypermutability. However, in our study the majority of epidemic strains studied were either hypo- or normomutable (47 % and 40 %, respectively). Only 2/15 (13 %) were strong mutators, suggesting that the phenomenon of epidemic strains cannot easily be explained by hypermutability. On closer examination, however, the situation proved to be complex. For example, strain E2331 from a patient whose first *P. aeruginosa* infection was with the Liverpool epidemic strain (LES) (Cheng *et al.*, 1996; McCallum *et al.*, 2001; Al-Aloul *et al.*, 2005; Salunkhe *et al.*, 2005) had a mutation frequency of 7.2×10^{-7}, indicating that this strain was a weak mutator. This contrasted with a mutation frequency of 3.9×10^{-9} for strain H129 (derived from a different patient, and one of the first known examples of LES; Table 1). *mutS* sequencing of E2331 (Table 3) revealed a point mutation (A187S), which had coincidentally been found by Hogardt *et al.* (2006), using complementation studies, to be a cause of hypermutability. The consequences of this result are twofold. First, it demonstrates that even weak mutators (as defined by phenotypic screening) can exhibit *mutS* mutations, thus raising the question of what defines a mutator. This issue has been discussed in depth in a recent review of mutator phenotypes by Hall & Henderson-Begg (2006). Although initially this study aimed to declare any strain with a mutation frequency >20 times that of PAO1 (estimated by Oliver *et al.*, 2000 to be approximately 4×10^{-7}) to be a hypermutator, the number of resistant PAO1 colonies on the rifampicin plates was found to be sufficiently variable so as to make comparison of mutation frequencies between individual experiments unfeasible. It was therefore decided that a standard value ($\geqslant 1 \times 10^{-6}$) be used. As a result of setting a high threshold, it is clear that the number of

hypermutators in this study may have been underestimated, a fact that has been highlighted by strain E2331. This result suggests that, in this study at least, weak mutators should also be considered to be potential hypermutators.

The second implication of the mutation frequency deviation between the two LES isolates is that it demonstrates the considerable intra-clonal variation exhibited by such strains, a characteristic elegantly demonstrated in a study by Salunkhe *et al.* (2005), who compared transcriptome profiles of two clones of LES from related patients. The fluctuation in mutation frequencies found in this study prompted a more in-depth analysis of these isolates. Screening of strains from individual patients infected with the Liverpool, Manchester, Bristol and Dublin group 3 epidemic strains revealed some interesting results, in particular a possible connection between mucoidy and mutation frequency within a clonal group. In addition, one of the Manchester epidemic strains had an unusually high mutation frequency compared to the other strains in this cluster. The variation in mutation frequencies does not appear to correlate with the stage of the outbreak, although this is difficult to prove without patient contact tracing. Rather, these results suggest that perhaps hypermutabilty in some of these epidemic strains is transient and is influenced by the state of an individual patient's lungs, by antibiotic treatment at the time, or by the complex interplay between the microbial population in the CF lung, as has been suggested to occur in other bacterial populations (Rosche & Foster, 1999; Blazquez, 2003). It would be interesting to look in more detail at some of these isolates to establish whether transient mutation really is responsible for these differences and to establish what, if any, connection there might be between mucoidy and mutation rate.

Interestingly, *mutS* mutations that might explain the raised mutation frequency were found in only three of the seven strongly hypermutable strains found in this study, and even these would need to be confirmed by complementation studies. This result suggests that, in *P. aeruginosa* at least, genes other than *mutS* are a more common cause of hypermutability than previously thought. Oliver *et al.* (2002) found that after *mutS*, *mutL* and *uvrD* are the next most common genes to be disrupted in mutator strains. In our study, whole-gene amplification of these, as well as the other mismatch repair and error prevention genes *mutY*, *mutM*, *mutD* and *mutT*, did not reveal any significant deletions. It seems likely therefore that a point mutation in one of these genes is responsible for the hypermutable phenotype of the other four strains in this study.

In conclusion, this study has revealed a higher than predicted level of both strong and weak mutators in early *P. aeruginosa* infection in CF. These results strengthen the argument for an early and aggressive approach to antimicrobial therapy. The existence of a comparable number of weak mutators in a small environmental population of *P. aeruginosa* suggests that the CF lung is not the only influential factor in the occurrence of mutators. In addition, phenotypic screening of a group of epidemic strains has revealed that although hypermutability exists, other, as-yet-unidentified, factors are likely to play a more important role in the success of these strains. Lastly, *mutS* sequencing of the strong hypermutators only found non-synonymous mutations in three of seven strains, suggesting that genes other than *mutS* are a more common cause of elevated mutation rates in *P. aeruginosa* than previously thought.

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REFERENCES

Al-Aloul, M., Miller, H., Stockton, P., Ledson, M. J. & Walshaw, M. J. (2005). Acute renal failure in CF patients chronically infected by the Liverpool epidemic *Pseudomonas aeruginosa* strain (LES). *J Cyst Fibros* **4**, 197–201.

Armstrong, D. S., Nixon, G. M., Carzino, R., Bigham, A., Carlin, J. B., Robins-Browne, R. M. & Grimwood, K. (2002). Detection of a widespread clone of *Pseudomonas aeruginosa* in a pediatric cystic fibrosis clinic. *Am J Respir Crit Care Med* **166**, 983–987.

Armstrong, D., Bell, S., Robinson, M., Bye, P., Rose, B., Harbour, C., Lee, C., Service, H., Nissen, M. & other authors (2003). Evidence for spread of a clonal strain of *Pseudomonas aeruginosa* among cystic fibrosis clinics. *J Clin Microbiol* **41**, 2266–2267.

Baquero, M.-R., Nilsson, A. I., del Carmen Turrientes, M., Sandvang, D., Galán, J. C., Martínez, J. L., Frimodt-Møller, N., Baquero, F. & Andersson, D. I. (2004). Polymorphic mutation frequencies in *Escherichia coli*: emergence of weak mutators in clinical isolates. *J Bacteriol* **186**, 5538–5542.

Blazquez, J. (2003). Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. *Clin Infect Dis* **37**, 1201–1209.

Burns, J. L., Gibson, R. L., McNamara, S., Yim, D., Emerson, J., Rosenfeld, M., Hiatt, P., McCoy, K., Castile, R. & other authors (2001). Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. *J Infect Dis* **183**, 444–452.

Butler, S. L., Doherty, C. J., Hughes, J. E., Nelson, J. W. & Govan, J. R. W. (1995). *Burkholderia cepacia* and cystic fibrosis: do natural environments present a potential hazard? *J Clin Microbiol* **33**, 1001–1004.

Chambers, D., Scott, F., Bangur, R., Davies, R., Lim, A., Walters, S., Smith, G., Pitt, T., Stableforth, D. & Honeybourne, D. (2005). Factors associated with infection by *Pseudomonas aeruginosa* in adult cystic fibrosis. *Eur Respir J* **26**, 651–656.

Chao, L. & Cox, E. C. (1983). Competition between high and low mutating strains of *Escherichia coli*. *Evolution* **37**, 125–134.

Cheng, K., Smyth, R. L., Govan, J. R. W., Doherty, C., Winstanley, C., Denning, N., Heaf, D. P., van Saene, H. & Hart, C. A. (1996). Spread of a β-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet* **348**, 639–642.

Ciofu, O., Riis, B., Pressler, T., Poulsen, H. E. & Hoiby, N. (2005). Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother* **49**, 2276–2282.

Curran, B., Morgan, J. A. W., Honeybourne, D. & Dowson, C. G. (2005). Commercial mushrooms and bean sprouts are a source of *Pseudomonas aeruginosa. J Clin Microbiol* **43**, 5830–5831.

Edenborough, F. P., Stone, H. R., Kelly, S. J., Zadik, P., Doherty, C. J. & Govan, J. R. W. (2004). Genotyping of *Pseudomonas aeruginosa* in cystic fibrosis suggests need for segregation. *J Cyst Fibros* **3**, 37–44.

Gibson, R. L., Burns, J. L. & Ramsey, B. W. (2003). Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* **168**, 918–951.

Goldberg, J. B. (2000). *Pseudomonas*: global bacteria. *Trends Microbiol* **8**, 55–57.

Gutiérrez, O., Juan, C., Pérez, J. L. & Oliver, A. (2004). Lack of association between hypermutation and antibiotic resistance development in *Pseudomonas aeruginosa* isolates from intensive care unit patients. *Antimicrob Agents Chemother* **48**, 3573–3575.

Hall, L. M. C. & Henderson-Begg, S. K. (2006). Hypermutable bacteria isolated from humans – a critical analysis. *Microbiology* **152**, 2505–2514.

Herman, G. E. & Modrich, P. (1981). *Escherichia coli* K12 clones that overproduce dam methylase are hypermutable. *J Bacteriol* **145**, 644–646.

Hogardt, M., Schubert, S., Adler, K., Götzfried, M. & Heesemann, J. (2006). Sequence variability and functional analysis of MutS of hypermutable *Pseudomonas aeruginosa* cystic fibrosis isolates. *Int J Med Microbiol* **296**, 313–320.

Jones, A. M., Govan, J. R. W., Doherty, C. J., Dodd, M. E., Isalska, B. J., Stanbridge, N. & Webb, A. K. (2001). Spread of a multi-resistant strain of *Pseudomonas aeruginosa* in an adult cystic fibrosis clinic. *Lancet* **358**, 557–558.

Jones, A. M., Govan, J. R. W., Doherty, C. J., Dodd, M. E., Isalska, B. J., Stanbridge, N. & Webb, A. K. (2003). Identification of airborne dissemination of epidemic multiresistant strains of *Pseudomonas aeruginosa* at a CF centre during a cross-infection outbreak. *Thorax* **58**, 525–527.

Kelly, N. M., Falkiner, F. R. & Keane, C. T. (1983). Acetamide broth for isolation of *Pseudomonas aeruginosa* from patients with cystic fibrosis *J Clin Microbiol* **17**, 159.

Maciá, M. D., Borrell, N., Pérez, J. L. & Oliver, A. (2004). Detection and susceptibility testing of hypermutable *Pseudomonas aeruginosa* with the Etest and disk diffusion. *Antimicrob Agents Chemother* **48**, 2665–2672.

Maciá, M. D., Blanquer, D., Togores, B., Sauleda, J., Pérez, J. L. & Oliver, A. (2005). Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrob Agents Chemother* **49**, 3382–3386.

Maciá, M. D., Borrell, N., Segura, M., Gómez, C., Pérez, J. L. & Oliver, A. (2006). Efficacy and potential for resistance selection of antipseudomonal treatments in a mouse model of lung infection by hypermutable *Pseudomonas aeruginosa. Antimicrob Agents Chemother* **50**, 975–983.

McCallum, S. J., Corkhill, J., Gallagher, M., Ledson, M. J., Hart, C. A. & Walshaw, M. J. (2001). Superinfection with a transmissible strain of *Pseudomonas aeruginosa* in adults with cystic fibrosis chronically colonised by *P. aeruginosa. Lancet* **358**, 558–560.

Obmolova, G., Ban, C., Hsieh, P. & Yang, W. (2000). Crystal structure of mismatch repair protein MutS and its complex with a substrate DNA. *Nature* **407**, 703–710.

Oliver, A., Canton, R., Campo, P., Baquero, F. & Blazquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288**, 1251–1253.

Oliver, A., Baquero, F. & Blázquez, J. (2002). The mismatch repair system (*mutS, mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterisation of naturally occurring mutants. *Mol Microbiol* **43**, 1641–1650.

Oliver, A., Levin, B. R., Juan, C., Baquero, F. & Blazquez, J. (2004). Hypermutation and the preexistence of antibiotic-resistant *Pseudomonas aeruginosa* mutants: implications for susceptibility testing and treatment of chronic infections. *Antimicrob Agents Chemother* **48**, 4226–4233.

Panagea, S., Winstanley, C., Walshaw, M. J., Ledson, M. J. & Hart, C. A. (2005). Environmental contamination with an epidemic strain of *Pseudomonas aeruginosa* in a Liverpool cystic fibrosis centre, and a study of its survival on dry surfaces. *J Hosp Infect* **59**, 102–107.

Prunier, A. L., Malbruny, B., Laurans, M., Brouard, J., Duhamel, J. F. & Leclercq, R. (2003). High rate of macrolide resistance in *Staphylococcus aureus* strains from patients with cystic fibrosis reveals high proportions of hypermutable strains. *J Infect Dis* **187**, 1709–1716.

Román, F., Canton, R., Perez-Vazquez, M., Baquero, F. & Campos, J. (2004). Dynamics of long-term colonisation of respiratory tract by *Haemophilus influenzae* in cystic fibrosis patients shows a marked increase in hypermutable strains. *J Clin Microbiol* **42**, 1450–1459.

Römling, U., Wingender, J., Müller, H. & Tümmler, B. (1994). A major cystic fibrosis clone common to patients and aquatic habitats. *Appl Environ Microbiol* **60**, 1734–1738.

Rosche, W. A. & Foster, P. L. (1999). The role of transient hypermutators in adaptive mutation in *Escherichia coli. Proc Natl Acad Sci U S A* **96**, 6862–6867.

Salunkhe, P., Smart, C. H., Morgan, J. A., Panagea, S., Walshaw, M. J., Hart, C. A., Geffers, R., Tummler, B. & Winstanley, C. (2005). A cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance. *J Bacteriol* **187**, 4908–4920.

Scott, F. W. & Pitt, T. L. (2004). Identification and characterisation of transmissible *Pseudomonas aeruginosa* strains in cystic fibrosis patients in England and Wales. *J Med Microbiol* **53**, 609–615.

Smith, E. E., Buckley, D. G., Wu, Z., Saeniphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P. & other authors (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A* **103**, 8487–8492.

Spilker, T., Coeyne, T., Vandamme, P. & LiPuma, J. J. (2004). PCR-based assay for the differentiation of *Pseudomonas aeruginosa* from other species of *Pseudomonas* recovered from cystic fibrosis patients. *J Clin Microbiol* **42**, 2074–2079.

Taylor, R. F., Morgan, D. W., Nicholson, P. S., Mackay, I. S., Hodson, M. E. & Pitt, T. L. (1992). Extrapulmonary sites of *Pseudomonas aeruginosa* in adults with cystic fibrosis. *Thorax* **47**, 426–428.

Watson, M. E., Jr, Burns, J. L. & Smith, A. L. (2004). Hypermutable *Haemophilus influenzae* with mutations in *mutS* are found in cystic fibrosis sputum. *Microbiology* **150**, 2947–2958.

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