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

Dervla T. Kenna,1 Catherine J. Doherty,1 Juliet Foweraker,2 Lisa Macaskill,3 Victoria A. Barcus1 and John R. W. Govan1

1Cystic Fibrosis Group, Centre for Infectious Diseases, University of Edinburgh Medical School, The Chancellor’s Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK
2Department of Microbiology, Papworth Hospital NHS Trust, Papworth Everard, Cambridge CB3 8RE, UK
3The Whitchurch Laboratory, Department of Microbiology, Faculty of Medicine, Nursing and Health Sciences, Monash University, Victoria 3800, Australia

*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.

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;
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; Ciouf 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 hypermutability 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^{-4}, 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 mutants (strains with a mutation frequency of 2×10^{-2} > f > 1×10^{-5}) and strong mutants (f ≥ 1×10^{-6}; approximately 20-fold greater than that of PAO1). Finally strains with f < 7×10^{-6} 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

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Epidemic</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H129</td>
<td>LES</td>
<td>Cheng et al. (1996)</td>
</tr>
<tr>
<td>C3425</td>
<td>MAN</td>
<td>Jones et al. (2001)</td>
</tr>
<tr>
<td>C4448</td>
<td>Clone C Germany, P10118</td>
<td>Römling et al. (1994)</td>
</tr>
<tr>
<td>C4269, C4270</td>
<td>Brisbane, Australia</td>
<td>Armstrong et al. (2003)</td>
</tr>
<tr>
<td>C3796, C3798</td>
<td>Melbourne, Australia</td>
<td>Armstrong et al. (2002)</td>
</tr>
<tr>
<td>E859</td>
<td>Shefield</td>
<td>Edenborough et al. (2004)</td>
</tr>
<tr>
<td>E1632</td>
<td>Yorkhill, Glasgow</td>
<td>Unpublished</td>
</tr>
<tr>
<td>E1476</td>
<td>Dublin 1</td>
<td>Unpublished</td>
</tr>
<tr>
<td>E1453</td>
<td>Dublin 2</td>
<td>Unpublished</td>
</tr>
<tr>
<td>E1457</td>
<td>Dublin 3</td>
<td>Unpublished</td>
</tr>
<tr>
<td>E1609</td>
<td>Dublin 4</td>
<td>Unpublished</td>
</tr>
<tr>
<td>E1284</td>
<td>Bristol</td>
<td>Unpublished</td>
</tr>
</tbody>
</table>

**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 MgCl₂ (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/).

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 mutants and 5/100 (5 %) were strong...
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 $9 \times 10^{-9}$ and $2 \times 10^{-7}$, 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 ($3.5 \times 10^{-5}$) 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

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 $7 \times 10^{-9}$ and $2 \times 10^{-7}$), weak mutators (with a mutation frequency $>2 \times 10^{-7}$ and $<1 \times 10^{-6}$), strong mutators ($\geq 1 \times 10^{-6}$) and hypomutable ($<7 \times 10^{-9}$). 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).
Table 3. Mutation frequencies and non-synonymous mutS mutations in strong mutators

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Mutation frequency</th>
<th>Amino acid position</th>
<th>Nucleotide base position</th>
<th>Codon from PAO1 (amino acid)</th>
<th>Codon change in mutator strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4649</td>
<td>Belgium, clinical</td>
<td>7.3 x 10^{-6}</td>
<td>398</td>
<td>1193 (4 bp insertion: GCAC)</td>
<td>ACC (Thr)</td>
<td>His inserted after Thr (ACG, then CAC)</td>
</tr>
<tr>
<td>C4662</td>
<td>Belgium, clinical</td>
<td>1.1 x 10^{-5}</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>C3182</td>
<td>Edinburgh (UK), clinical</td>
<td>2.5 x 10^{-6}</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>C4488</td>
<td>Manchester (UK), clinical</td>
<td>2.5 x 10^{-5}</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>E2889</td>
<td>Paisley (UK), clinical</td>
<td>1.7 x 10^{-6}</td>
<td>636</td>
<td>1907</td>
<td>GGC (Gly)</td>
<td>GAC (Asp)</td>
</tr>
<tr>
<td>E1284</td>
<td>Bristol (UK), clinical</td>
<td>6.3 x 10^{-6}</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>E1457</td>
<td>Dublin (RoI), clinical</td>
<td>5.7 x 10^{-6}</td>
<td>545</td>
<td>1633</td>
<td>GGC (Ala)</td>
<td>ACC (Thr)</td>
</tr>
<tr>
<td>E2331*</td>
<td>Papworth (epidemic)</td>
<td>7.2 x 10^{-7}</td>
<td>187</td>
<td>2078</td>
<td>GAG (Glu)</td>
<td>GGG (Gly)</td>
</tr>
</tbody>
</table>

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 hypermutable 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...
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; Macià 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. Macià 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 (≥1 × 10⁻⁶) be used. As a result of setting a high threshold, it is clear that the number of

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

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

*The unusually high mutation frequency of this strain was reproducible.
†These isolates had a mucoid phenotype.
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 hypermutability 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 mutT, mutM, mutD and mutU, 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.

ACKNOWLEDGEMENTS

We would like to thank Mr Fraser Pike and Miss Lisa O’Gorman for technical assistance and Dr Diane Bilton and Mrs Christian Laughton (Papworth Hospital, UK) for their collaboration on this project. We would also like to thank Dr Ty Pitt for the gift of the mutator strain P. aeruginosa J3295 and the Hanover epidemic strain, clone C. The Brisbane and Melbourne strains were kindly provided by Dr David Armstrong, Royal Children’s Hospital, Melbourne. Dr Andrew Jones (Wythenshawe Hospital, Manchester, UK) and Professor Mario Vanechoutte (University Hospital, Gent, Belgium) provided some of the early isolates for this study and Professor Peter Vandamme (Universiteit Gent, Belgium) some of the environmental strains. Finally, we acknowledge the services of the ICMB sequencing service (University of Edinburgh), and Chiron for financial support.

REFERENCES


Edited by: P. Cornelis