Emergence of phenotypic variants upon mismatch repair disruption in *Pseudomonas aeruginosa*

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MutS is part of the bacterial mismatch repair system that corrects point mutations and small insertions/deletions that fail to be proof-read by DNA polymerase activity. In this work it is shown that the disruption of the *P. aeruginosa* mutS gene generates the emergence of diverse colony morphologies in contrast with its parental wild-type strain that displayed monomorphic colonies. Interestingly, two of the mutS morphotypes emerged at a high frequency and in a reproducible way and were selected for subsequent characterization. One of them displayed a nearly wild-type morphology while the other notably showed, compared with the wild-type strain, increased production of pyocyanin and pyoverdin, lower excretion of LasB protease and novel motility characteristics, mainly related to swarming. Furthermore, it was reproducibly observed that, after prolonged incubation in liquid culture, the pigmented variant consistently emerged from the mutS wild-type-like variant displaying a reproducible event. It is also shown that these *P. aeruginosa* mutS morphotypes not only displayed an increase in the frequency of antibiotic-resistant mutants, as described for clinical *P. aeruginosa* mutator isolates, but also generated mutants whose antibiotic-resistant levels were higher than those measured from spontaneous resistant mutants derived from wild-type cells. It was also found that both morphotypes showed a decreased cytotoxic capacity compared to the wild-type strain, leading to the emergence of invasive variants. By using mutated versions of a tetracycline resistance gene, the mutS mutant showed a 70-fold increase in the reversion frequency of a +1 frameshift mutation with respect to its parental wild-type strain, allowing the suggestion that the phenotypical diversity generated in the mutS population could be produced in part by frameshift mutations. Finally, since morphotypical diversification has also been described in clinical isolates, the possibility that this mutS diversification was related to the high frequency hypermutability observed in *P. aeruginosa* CF isolates is discussed.

INTRODUCTION

MutS is one of the main components of the mismatch repair system (MMRS), which monitors the fidelity of DNA replication and recombination by repairing DNA polymerase errors and blocking homologous recombination events (Bueremyer *et al.*, 1999; Fishel, 1998; Horst *et al.*, 1999; Modrich & Lahue, 1996). Consequently, MMRS-defective bacteria show both an enhanced rate of mutation and an increase in recombination of divergent sequences (Modrich & Lahue, 1996).

In *Escherichia coli*, it has been generally proposed that the MMRS is involved in the regulation of adaptive strategies, controlling the mutation and recombination frequency that leads to genetic diversification (Giraud *et al.*, 2001; Taddei *et al.*, 1997; Rayssiguier *et al.*, 1989). As a consequence, the presence of mutator strains in a cell population, capable of generating rare favourable mutations at a higher rate, could be beneficial to allow both evolution and adaptation to new and changing environments (Horst *et al.*, 1999; Taddei *et al.*, 1997). In this sense, several reports have proposed that survival strategies, such as phase variation (Henderson *et al.*, 1999; Robertson & Meyer, 1992) and pathoadaptive mutations might be accelerated in bacterial
clones that are transient or genetically stable mutators (Richardson & Stojilikovic, 2001; Sokurenko et al., 1998, 1999; Bucci et al., 1999).

In recent years, the study of the MMR system in the versatile Pseudomonas aeruginosa (Oliver et al., 2000, 2002; Pezza et al., 2002a, b; Kresse et al., 2003) revealed that this species constitutes an interesting model to understand the role of this system in bacterial adaptive strategies. The clinical relevance of P. aeruginosa is largely known as it is an important human opportunistic pathogen, frequently involved in severe and often fatal infections in patients with cystic fibrosis (CF) and other immunodefensive illnesses (Govan & Deretic, 1996; van Delden & Iglewski, 1998). An interesting observation is that, particularly from CF patients, P. aeruginosa isolates display significant phenotypic variation, such as a wide spectrum of colony variants, development of mucoid phenotype and highly adherent small-colony variants, absence of cell motility, development of variants resistant to macrophage phagocytosis and acquisition of resistance to multiple antibiotics (Deretic et al., 1994; Govan & Deretic, 1996; Mahenthiralingam et al., 1994; Oliver et al., 2000; Häussler et al., 2003).

The relationship between mutator phenotypes and P. aeruginosa adaptability has been demonstrated by the observation of a high proportion of mutator P. aeruginosa isolates from CF patients (Oliver et al., 2000). In contrast, P. aeruginosa mutators were not present in acute infections, reflecting the particularity of the CF lung habitat (Oliver et al., 2000). At the molecular level, most of the P. aeruginosa mutator cells were found to be deficient in MMRS, predominantly with mutations in the mutS gene (Kresse et al., 2003). These studies suggest that increased mutation and homologous recombination rates would facilitate the appearance of P. aeruginosa phenotypes able to adapt to the heterogeneous and changing environment present in the CF lung.

Bacterial strains that exhibit elevated mutation frequencies have also been reported among populations of pathogenic E. coli, Salmonella enterica and Neisseria meningitidis (Denamur et al., 2002; LeClerc et al., 1996; Bucci et al., 1999), strengthening the hypothesis that a mutator phenotype would play an important role in the emergence of pathogenic strains (Taddei et al., 1997; McKenzie & Rosenberg, 2001; Metzgar & Wills, 2000; Oliver et al., 2000).

Despite the potential importance of mutator strains with respect to adaptive evolution, the phenotypic consequences of the hypermutability in P. aeruginosa have been poorly examined. To obtain new information in this aspect, we have performed a phenotypic characterization of a P. aeruginosa Hex1T mutator strain generated by the knockout of the mutS gene that inactivates the MMRS and leads to a 100- to 1000-fold mutation rate increase with respect to its parental wild-type strain. In this work we describe that after a relatively short period of cultivation, morphological colony variants emerged from the P. aeruginosa Hex1T mutS mutant. In turn, these variants were associated with a wide spectrum of phenotypes related to several phenotypic traits, including virulence, suggesting that the acquisition of certain phenotypes would be accelerated in a mutator cell and could favour adaptive divergence processes. Furthermore, according to our results, we speculate that the phenotypic variability of the mutS strain could be generated by mutations in specific genes probably by means of a frameshift mechanism.

**METHODS**

**Bacterial strains and media.** P. aeruginosa Hex1T used in this work was originally isolated from hydrocarbon-contaminated soil (Pezza et al., 2002a). This bacterium was typed by conventional microbiological tests and by sequencing a DNA fragment of the 16S RNA. Sequencing results indicated a 99% identity with the corresponding PAO1 16S DNA fragment.

For the P. aeruginosa mutS mutant construction the following E. coli strains were used: DH5α [supE44 ΔlacU169 (Δ80lacZAM15) hisD17 recA1 endA1 gyr96 thi-1 relA1] and CC118 (ispA [A(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1 Δ(π phage lysogen)]. Both E. coli and P. aeruginosa strains were routinely subcultured on Luria–Bertani (LB) agar plates from glycerol frozen stocks. The inoculum was prepared using an overnight culture in LB broth at 30 °C (P. aeruginosa) or 37 °C (E. coli) with appropriate aeration. For pyocyanin and pyoverdine assays, P. aeruginosa strains were grown on 1% NaCl, 1% soy peptone and 0-5% yeast extract (LBS), or King A or King B liquid media (King et al., 1954). For biofilm determination the cultures were carried out in tryptose-soy (TS) medium. Antibiotics were used at the following concentrations unless otherwise indicated: ampicillin, 100 µg ml⁻¹; rifampicin, 100 µg ml⁻¹; kanamycin, 200 µg ml⁻¹.

**Construction of plasmid pKNG101-mutS::kan.** The generation of the P. aeruginosa mutS mutant was carried out by replacing the endogenous mutS gene with a disrupted mutS::kan allele subcloned in the mobilizable suicide plasmid pKNG101 (Kariga et al., 1991). To construct the plasmid pKNG101-mutS::kan, the plasmid pBlueScript-mutS (Pezza et al., 2002a) containing the P. aeruginosa mutS gene on a 3-9 kb BamHI–HindIII fragment was digested with PsI and EcoRI to delete approximately 1-7 kb within the mutS gene. The deleted region was then replaced with a 1-8 kb fragment encoding the kanamycin (Km) resistance gene obtained from pUTminiTn5-kan (de Lorenzo & Timmis, 1994). The resulting cassette (mutS::kan) contains the Km resistance gene flanked by mutS sequences on both sides. Using Sphi and Apol present in the pBlueScript polylinker site, the mutS::kan cassette was then subcloned into pKNG101 and the resulting plasmid was used to deliver the mutS::kan allele to the host chromosome via homologous recombination.

**Plasmid conjugation.** Triparental mating was performed following protocols described by de Lorenzo & Timmis (1994) using E. coli HB101 RK6 as helper strain, CC118 (ispA) transformed with pKNG101-mutS::kan as donor strain and P. aeruginosa Hex1T as recipient strain. For conjugation, 100 µl of overnight cultures of donor, helper and recipient strains were mixed in 5 ml 10 mM MgSO₄ and collected on a Millipore filter which was subsequently placed on an LB agar plate and incubated overnight at 30 °C. After incubation, the cells were resuspended in 5 ml 10 mM MgSO₄ and
several aliquots were plated on M9 selective plates supplemented with Km and streptomycin (Sm), with and without 8% sucrose as described previously (Kaniga et al., 1991). Once the transconjugants (Km’ Su’ Sm’) were isolated, the mutator phenotype was detected by scoring resistant cells using rifampicin (100 μg ml−1) in LB agar plates.

**Determination of antibiotic-resistant cell frequency.** The measurement of the frequency of cells resistant for each antibiotic was determined for *P. aeruginosa* Hex1T wild-type and mutS mS1 and mS2 morphotypic variants. Briefly, one bacterial colony was suspended in 2 ml LB and grown at 30 °C overnight. About 10⁶ cells from these cultures were plated on LB agar plates to determine the number of viable cells, as well as on LB agar plates supplemented with 10 μg amikacin ml⁻¹, 10 μg gentamicin ml⁻¹ and 1 μg ciprofloxacin ml⁻¹ and 5 μg tobramycin ml⁻¹ to score for resistant cells. Following overnight incubation at 30 °C, the number of c.f.u. was measured and the resistance frequency for each antibiotic was determined as resistant cells per 10⁶ cells.

To determine the antibiotic resistance level of mutS and wild-type resistant cells, five independent colonies, one each resistant to 1 μg ciprofloxacin ml⁻¹, 10 μg gentamicin ml⁻¹ and 5 μg tobramycin ml⁻¹, were isolated and cultured overnight at 30 °C in LB in the presence of equal antibiotic concentrations. Each clone was then incubated in the presence of different concentrations of antibiotics as follows: 1, 2, 4 and 8 μg ciprofloxacin ml⁻¹; 10, 20, 40 and 80 μg gentamicin ml⁻¹, and 5, 10, 15 and 20 μg tobramycin ml⁻¹. Following overnight incubation at 30 °C with aeration, the OD₅₃₀ was determined.

**Determination of alginate production.** For the determination of alginate, *P. aeruginosa* Hex1T wild-type and mutS mS1 and mS2 variants were grown on LB supplemented with 0.2% glycerol at 30 °C for 72 h without shaking. After incubation, the cells were separated from the medium by centrifugation at 7000 g for 15 min and the alginate present in the supernatant was precipitated with 3 vols ethanol at −70 °C for 24 h followed by centrifugation at 18 000 g for 15 min. The pellet was resuspended in water and the alginate was quantified by the carbazol assay (Knutson & Jeanes, 1968) using gluconic acid as standard.

**Biofilm formation.** Biofilm formation was measured as described by Christensen et al. (1982) by cell adherence to a glass surface. Briefly, *P. aeruginosa* Hex1T wild-type and mutS mS1 and mS2 variants were grown in glass tubes containing TS medium supplemented with 0.25% glucose at 30 °C for 72 h without shaking. After incubation the cultures were removed and the tubes were washed three times with PBS to remove non-associated bacteria. Then, gentamicin (100 μg ml⁻¹) was added and the cells were incubated for an additional 2 h. The antibiotic was removed by three washes with PBS and, after inoculation with 0.3% Triton X-100 for 5 min, the cellular lysates were seeded in LB agar plates to determine the number of surviving intracellular bacteria. Three wells of cells were used for each strain and the experiment was carried out three times.

**Cytotoxicity assays.** Cytotoxicity was assessed by a trypan blue dye exclusion assay. MDCK cells were cultured as described above and grown overnight to approximately 80% confluence. Unlabelled epithelial cells were then incubated with approximately 3 x 10⁵ bacteria in suspension from *P. aeruginosa mutS* mS1 and mS2 and wild-type strains. Following a 3 h incubation period at 37 °C, the inoculum was removed and the cells were washed three times with PBS to remove non-associated bacteria. Then, gentamicin (100 μg ml⁻¹) was added and the cells were incubated for an additional 2 h. The antibiotic was removed by three washes with PBS and, after incubation with 0.3% Triton X-100 for 5 min, the cellular lysates were seeded in LB agar plates to determine the number of surviving intracellular bacteria. Three wells of cells were used for each strain and the experiment was carried out three times.

**Determination of tetracycline resistance reversion frequency.** To examine the reversion of mutant tetracycline resistance genes (tet genes) we constructed three plasmids for use as mutation targets. Different tet gene variants each containing different frameshift mutations that inactivate the tetracycline gene (see Table 3) were amplified by PCR from plasmids pW17, pW18 and pX2 (Torkelson et al., 1997). The amplification products were subcloned in the pGEM-T Easy vector (Promega) and subsequently cloned into the EcoRI– HindIII cloning sites of pBR1MC-5/Gentamicin (Kovach & Peterson, 1995), which can replicate in *Pseudomonas*. Each plasmid variant (named pBRR1-W17, pBRR1-W18 and pBRR1-X2) was finally introduced into *P. aeruginosa* mutS and wild-type strains by electroporation and the transformed cells isolated were gentamicin-resistant. For tetracycline resistance reversion analyses, mutS and finally with 10 vols water. The relative amount of pyoverdine in both the final extract of LBS and King B supernatants was quantified by measuring A₅₃₀.

**Determination of elastase activity.** Elastase (LasB protease) activity was determined in liquid cultures by the elastin-Congo red hydrolysis assay as described by Aendeker et al. (2002). All measurements were carried out in duplicate.

**Rhamnolipid detection.** The detection of secreted rhamnolipids by the *P. aeruginosa* mutS variants and wild-type strain was carried out as described by Siegmund & Wagner (1991). Briefly, 10 μl of an overnight LB culture of the different *P. aeruginosa* strains was plated on M9 minimal medium agar plates containing 0.5% glutamate, 0.2 g CTAB (cetyltrimethylammoniumbromide) l⁻¹ and 5 mg methylene blue l⁻¹. Following 48 h of incubation at 30 °C, the diameter of the clearing zones around the inocula was measured.

**Motility assays.** Swimming, swarming and twitching motility were assessed according to Deziel et al. (2001), except that the plates were incubated at 30 °C for 48–72 h and in the twitching motility assay Coomassie blue was used instead of crystal violet.

**Determination of eukaryotic cell invasion by *P. aeruginosa* wild-type and mutS variants.** The capacity of *P. aeruginosa* Hex1T wild-type and the mutS mS1 and mS2 variants to invade epithelial Madin–Darby canine kidney (MDCK) cells was determined as described by Alonso et al. (1999). Briefly, MDCK cells were cultured in 12-well tissue-culture plates in Eagle minimal essential medium supplemented with 10% fetal bovine serum and used for experiments after 24 h growth (around 70–80% confluence; approximately 2.5 x 10⁵ cells per well). Cells were inoculated with *P. aeruginosa* wild-type or the mutS variants by the addition of 50 μl of a bacterial suspension containing 3 x 10⁶ cells. Following a 2 h incubation at 37 °C, the inoculum was removed and the cells were washed three times with PBS to remove non-associated bacteria. Then, gentamicin (100 μg ml⁻¹) was added and the cells were incubated for an additional 2 h. The antibiotic was removed by three washes with PBS and, after incubation with 0.3% Triton X-100 for 5 min, the cellular lysates were seeded in LB agar plates to determine the number of surviving intracellular bacteria. Three wells of cells were used for each strain and the experiment was carried out three times.

**Pyocyanin quantification.** To determine the pyocyanin pigment, *P. aeruginosa* Hex1T wild-type and mutS mS1 and mS2 variants were grown for 48 h at 30 °C without shaking in both LBS and King A media (King et al., 1994). Cells were discarded from LBS medium by centrifugation at 7000 g for 15 min and the pyocyanin present in the supernatant was extracted with 1 vol. chloroform and 1 vol. 0.2 M HCL. The pigment was quantified by A₅₃₀. Supernatants from King A medium were also obtained by centrifugation at 7000 g for 15 min and the relative concentration of pyocyanin was measured spectrophotometrically at 695 nm.

**Pyoverdin quantification.** For pyoverdin determination *P. aeruginosa* Hex1T wild-type and mutS mS1 and mS2 variants were grown for 48 h at 30 °C without shaking in both LBS and King B medium (King et al., 1994). Cells were discarded by centrifugation at 7000 g for 15 min. The supernatant from LBS medium was successively extracted with 2 vols phenol/chloroform (1:1, v/v), 2 vols ether and
wild-type cells derived from strains carrying each of the plasmid variants were grown overnight on LB medium containing 20 μg gentamicin ml⁻¹. These cultures were then plated on LB agar plates to determine the number of viable cells, as well as on LB agar containing 55 μg tetracycline ml⁻¹ to determine the tetracycline revertant cells. Following overnight incubation at 30 °C, the tetracycline-resistant colonies were quantified and the frequency of tetracycline revertants was determined as the number of resistant cells per 10⁸ cells.

RESULTS

Construction of the P. aeruginosa mutS mutant

The postulated importance of mutator strains with respect to adaptive evolution and pathogenesis prompted the creation of a mutS knockout mutation in P. aeruginosa Hex1T to examine its phenotypic features, including those related to virulence. To generate the P. aeruginosa mutS mutant, a null P. aeruginosa mutS allele was constructed in a mobilizable suicide plasmid, pKNG101 (Caniga et al., 1991), by replacing the 1.7 kb PstI–EcoRI fragment of the mutS coding sequence by a 1.8 kb PstI–EcoRI fragment containing the Km resistance gene obtained from the pUT-miniTn5-Km vector (see Methods). After conjugation, P. aeruginosa transconjugants selected as Km resistant were isolated and by scoring the frequency of rifampicin-resistant cell mutants, we found in some of them a mutation frequency 100- to 1000-fold higher than in the wild-type strain, a fact that indicates the generation of a mutator phenotype. Molecular confirmation of mutS allele disruption in these mutator clones was carried out by Southern blotting (not shown).

Emergence of morphotypic variants correlates with mutS inactivation

As a way to investigate the evolution and stability of the mutS strain, we analysed the morphology of colony growth on LB agar after several periods of incubation in LB liquid medium. For this purpose individual mutS transconjugant colonies were used to inoculate liquid LB medium which was incubated overnight at 30 °C. Starting with this culture, three successive overnight LB liquid cultures were set up using 1/100 dilution of the previous culture as inoculum. Strikingly, we observed that at this stage colonies with different morphology emerged. To further characterize mutS colony diversification, aliquots from this culture were frozen at −70 °C in the presence of 15% glycerol. These frozen cells were the starting stock used for the rest of the experiments.

It is important to point out that under the same growth conditions, the wild-type P. aeruginosa Hex1T parental strain showed monomorphic colonies, always convex and circular with a diameter of ~3 mm (Fig. 1a). However, while one of the mutS morphotypes (Fig. 1b) formed slightly smaller (~2.5 mm diam. after 48 h at 30 °C) convex and circular colonies similar to the wild-type morphology (hereafter referred to as mS1 morph), the colonies of the other morphotype (Fig. 1c) were more transparent, 3-5 mm in size with a flat periphery usually spreading out around the colony (hereafter referred to as mS2 morph). Furthermore, after several days of incubation at 30 °C or at room temperature, these flatter extensions notably formed a ribbed surface (Fig. 1e). Another clear difference between mS1 and mS2 variants was that on LB agar plates all mS2 colonies appeared uniformly pigmented (Fig. 1c) due to pyoverdine and pyocyanin production (see below). Moreover, co-cultivation of mS2 with wild-type or mS1 colonies showed that the mS2 pigmentation is not altered and it can be used to differentiate this morphotype in a heterogeneous mixture of cells (Fig. 1f).

A third kind of colony (mS3; Fig. 1d) was sporadically observed which looks similar to the ‘spontaneous lysing colonies’ described by D’Argenio et al. (2002). However, compared to the mS1 and mS2 morphotypes which represented approximately 60 and 40%, respectively, of the total colonies, the mS3 morphotype appeared very rarely and its appearance was not reproducible; for this reason this morphotype was not further characterized.

Growth behaviour and fate of mutS morphotypic variants

Once recognized, the two mutS morphotypic variants were isolated (Fig. 1) and their growth behaviour with respect to the wild-type strain was analysed. When the isolated morphotype variants were grown in agitated LB liquid medium, we observed that in the exponential phase they grew approximately twofold slower than the wild-type strain. However, the two morphotypes reached almost equal density at stationary phase with respect to the wild-type (not shown). Equally, when the mutS morphotypic variants were grown on LB agar medium, although the colonies developed later in comparison to the wild-type ones, they continued to grow to an equivalent size. Also, performing prolonged culture experiments in agitated LB medium, we observed that both mS1 and mS2 variants displayed equivalent capacity to survive in late stationary phase relative to the wild-type. Furthermore, after 6 days incubation, the number of c.f.u. of mS1 and mS2 exceeded the wild-type by approximately 1.5- and 3-fold, respectively. Another consistent observation was that after approximately 120 h incubation, while the wild-type and the mS2 variant did not reveal any apparent changes in colony morphology, the mS1 diversified into the mS2 morph, suggesting that mS1 is a precursor to the mS2 variant.

Expression of several virulence factors are altered in mutS morphotypic variants

Since motility is intimately involved in diverse processes such as symbiosis, biofilm formation and virulence, the various forms of P. aeruginosa motility were examined in the mutS morphotypic variants and their parental wild-type strain. We observed that although on swimming agar plates mS2 did not exhibit significant differences with...
respect to the wild-type (Fig. 2), on swarming agar plates it displayed an increased zone of motility within 48–72 h in comparison to the wild-type. After 72 h incubation, the mean diameter of the mS2 was 2.6 ± 0.15 cm, whereas the mean diameter of the wild-type was smaller (1.5 ± 0.1 cm). Again, on swarming plates mS2 showed a characteristic way of spreading by flatter extensions with ridges (Fig. 2). Regarding the mS1 variant, we observed a similar but slightly impaired motility not only on swimming but also on swarming agar plates (mean diameter = 1.1 ± 0.07 cm; Fig. 2). Finally, examining the twitching motility capability of the wild-type and mutS morphotypes, we observed that while the wild-type and mS1 morph showed a motility zone of circular shape, the mS2 morph displayed an irregular edged motility zone and the development of bright radial zones different to that observed in the wild-type strain (Fig. 2). Additionally, the mS2 surface colonies on top of the agar were larger in size and irregular in shape with respect to the mS1 and wild-type strains.

The analysis of secondary metabolite production, known to be important factors for P. aeruginosa virulence, also revealed a clear heterogeneity between the mutS variants. Determination of pyocyanin production showed that compared with the wild-type strain, the mS2 morph produces 3–12 times more pyocyanin (Table 1). Furthermore, culture supernatants of the mS2 morph contained 2–2.5 times more pyoverdin than its wild-type counterpart (Table 1). Despite its lower values, the mS1 variant also showed differences with respect to the wild-type strain (Table 1). This hyper-pigmentation was notably expressed in particular media such as LBS and King A or King B, whereas in other media it was less evident (not shown). Furthermore, the hyper-pigmentation was more evident in liquid cultures incubated without shaking.

Regarding rhamnolipid production, we observed slightly higher values for the mS2 variant than for the wild-type

**Fig. 1.** Morphological diversity exhibited by P. aeruginosa mutS colonies. To isolate the different morphotypes, mutS cells from the frozen stock were grown on LB agar overnight at 30 °C and single colonies of each morphotype were streaked again on LB agar and grown for 48 h at 30 °C. (a) Wild-type strain; (b) mS1 wild-type-like morphotype; (c) mS2 pigmented morphotype; (d) mS3 ‘lysing’-like colonies; (e) mS2 at higher magnification after 72 h incubation at 30 °C; (f) a mixture of wild-type and mS2 variant.
The **mutS** morphotypic variants show a higher frequency of antibiotic-resistant cells and generate mutants with a higher level of antibiotic resistance

Antibiotic resistance remarkably contributes to pathogenesis and can be acquired by mutational mechanisms that can be induced by stressful environments (Martinez & Baquero, 2000, 2002). Particularly in *P. aeruginosa* an association between mutator strains and antibiotic resistance has been established in strains isolated from CF patients (Oliver *et al.*, 2000).

**Table 1.** Phenotypic comparison of *P. aeruginosa* *mutS* morphotypic variants and wild-type strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pyocyanin</th>
<th>Pyoverdin§</th>
<th>Rhamnolipids§</th>
<th>Elastase‖</th>
<th>Alginate¶</th>
<th>Biofilm§</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>LBS*</td>
<td>King A†</td>
<td>LBS</td>
<td>King B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.020 ± 0.001</td>
<td>0.030 ± 0.001</td>
<td>0.70 ± 0.07</td>
<td>0.45 ± 0.05</td>
<td>1.17 ± 0.06</td>
<td>0.70 ± 0.075</td>
</tr>
<tr>
<td><em>mutS</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mS1</td>
<td>0.080 ± 0.010</td>
<td>0.025 ± 0.002</td>
<td>0.80 ± 0.01</td>
<td>0.60 ± 0.06</td>
<td>1.40 ± 0.16</td>
<td>0.62 ± 0.080</td>
</tr>
<tr>
<td>mS2</td>
<td>0.235 ± 0.020</td>
<td>0.090 ± 0.005</td>
<td>1.60 ± 0.14</td>
<td>1.00 ± 0.08</td>
<td>1.74 ± 0.07</td>
<td>0.01 ± 0.005</td>
</tr>
</tbody>
</table>

*OD measured at 530 nm.
†OD measured at 695 nm (mean of two replicates).
‡OD measured at 350 nm (mean of two replicates).
§Relationship between the diameter of the clearing zones around the bacterial spots and the diameter of the bacterial spots in cm (mean of four replicates).
‖OD measured at 495 nm (mean of three replicates).
¶Determined by the carbazol assay (see Methods) and expressed as μg uronic acid (ml culture supernatant)^−1.
§OD of the films adhered to the glass and stained with safranine measured at 530 nm (mean of two replicates).
In the light of these observations, we analysed the level of antibiotic resistance of the *P. aeruginosa* mutS morphotypic variants, measuring the frequency of cells resistant to antibiotics commonly used in CF therapy. Using MICs of gentamicin, amikacin, ciprofloxacin and tobramycin we determined the frequency of mutant resistant colonies for *mutS* and wild-type strains. We found that both *mutS* variants behaved very similarly with all antibiotics tested and each of them showed approximately 50- (tobramycin), 215- (amikacin), 250- (ciprofloxacin) and 1070-fold (gentamicin) higher numbers of resistant cells compared with the wild-type (Table 2).

Furthermore, we analysed whether the *mutS* and wild-type antibiotic-resistant spontaneous mutants isolated from the previous assay generate mutants resistant to higher antibiotic concentrations in a subsequent round of selection with a higher level of antibiotic. By measuring the cell density (OD600) of overnight cultures grown in the presence with a higher level of antibiotic. By measuring the cell biotic concentrations in a subsequent round of selection previous assay generate mutants resistant to higher anti-antibiotic-resistant spontaneous mutants isolated from the furthermore, we analysed whether the *mutS* gene produces the emergence of invasive variants with impaired cytotoxicity

Previous studies have indicated that *P. aeruginosa* isolates can be broadly differentiated into two groups, expressing either a cytotoxic or an invasive phenotype in epithelial cells, which can also be associated with acute and chronic infection, respectively (Fleischig et al., 1994, 1997).

To further investigate the phenotypic differences between the *P. aeruginosa* mutS variants and their parent strain, we examined the ability of these strains to invade MDCK cells by a gentamicin survival assay (see Methods). As shown in Table 3, after 2 h incubation of MDCK cells with 3 x 10^6 bacterial cells, both *P. aeruginosa* mutS variants, mS1 and mS2, invaded at least 9-6- and 17-8-fold more efficiently than the wild-type strain, respectively. Interestingly, these differences were notably higher when the incubation was carried out with 10 times more bacteria (results not shown). Analysing these results, we observed that whereas for the *mutS* variants the increase in the number of bacteria correlated with the number of internalized ones, for the wild-type strain there was no such correlation and, in contrast, the number of internalized bacteria decreased with the increment of added bacteria in the incubation period (not shown). This result indicated that the *P. aeruginosa* wild-type was a cytotoxic strain and allowed us to speculate that the *mutS* disruption and the consequent elevated mutation frequency generated mutants within the population that had lost their cytotoxicity and thus became invasive. To analyse this possibility, we examined the cytotoxic capability for the wild-type strain and the two *mutS* variants by a trypan blue exclusion assay, which reveals eukaryotic cell death. As shown in Fig. 3, there was a significant difference in the percentage of stained cells among the *mutS* variants and wild-type strain. After 3 h incubation, the cells incubated with the wild-type *P. aeruginosa* strain showed nearly 80% stained cells, whereas those incubated with each of the *mutS* variants displayed only 24–27% (Fig. 4). This result confirmed the cytotoxic capability of the wild-type strain and clearly showed that in the *mutS* population variants emerge which display a non-cytotoxic phenotype and consequently an invasive capability.

### Disruption of the *mutS* gene produces the emergence of invasive variants with impaired cytotoxicity

Table 2. Determination of the number of *P. aeruginosa* Hex1T wild-type and *mutS* cells resistant to different antibiotics

The frequency of resistant cells was determined for gentamicin (10 μg ml^-1), amikacin (10 μg ml^-1), ciprofloxacin (1 μg ml^-1) and tobramycin (5 μg ml^-1). The antibiotic concentrations were below the MIC of each antibiotic. The experiments were carried out using the mutator assay (see Methods). Frequencies were determined for gentamicin, ciprofloxacin and tobramycin (see Methods) we found that the *mutS* clones displayed great variability in their resistance for all antibiotics tested, some of them displaying levels of significantly high resistance.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of antibiotic-resistant cells per 10^8 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>mS1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>32.6 ± 7.4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>10.7 ± 5.0</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>13.8 ± 1.0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>145.1 ± 14.0</td>
</tr>
<tr>
<td>mS2</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>32.870 ± 8.700</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2.880 ± 1.300</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>714 ± 140</td>
</tr>
<tr>
<td>Amikacin</td>
<td>29.600 ± 5.800</td>
</tr>
</tbody>
</table>

Table 3. Invasion of epithelial MDCK cells by *P. aeruginosa* mutS mS1 and mS2 variants and wild-type strain

Extracellular bacteria are those that remain in the cell culture medium after 2 h incubation with 100 μg gentamicin ml^-3 (see Methods). Intracellular bacteria indicates the viable bacteria recovered from the inside of the epithelial cells after a 2 h period of infection. The values are expressed as total c.f.u. per 10^8 added bacteria.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Extracellular bacteria</th>
<th>Intracellular bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>mS1</td>
<td>40 ± 8</td>
<td>604 ± 115</td>
</tr>
<tr>
<td>mS2</td>
<td>65 ± 9</td>
<td>1120 ± 187</td>
</tr>
<tr>
<td>Wild-type</td>
<td>5 ± 2</td>
<td>63 ± 10</td>
</tr>
</tbody>
</table>
The *P. aeruginosa* Hex1T mutS mutant showed a higher reversion frequency for +1 frameshift mutations

It is well known that many bacterial pathogens use strand-slippage as a regulatory mechanism to turn on/off various different genes involved in certain pathogenic processes, enabling bacterial populations to generate substantial phenotypic diversity in a short number of generations (Henderson et al., 1999; Moxon et al., 1994; Sokurenko et al., 1998). In addition, it was postulated that these ‘pathoadaptive’ mutations might be accelerated in bacterial clones that are transient or genetically stable mutators (Richardson & Stojiljkovic, 2001).

To analyse the putative underlying molecular mechanism that gives rise to the observed phenotypes, we determined the frequency of frameshift mutations produced in *P. aeruginosa*, comparing the mutS and wild-type strains. Using three plasmid-encoded mutated variants of a tetracycline resistance gene (Torkelson et al., 1997; see Methods), we examined the frequency of reversion to tetracycline resistance for the mutS and wild-type strains. The tetracycline genes contained different frameshift mutations that produced a truncated inactive protein, a +1 frameshift in a run of six G residues (pBBR1-W17), a −1 frameshift in the same G repeat (pBBR1-W18) and a GC insertion in a GC dinucleotide repeat (pBBR1-X2). The mutS and wild-type strains were transformed with each plasmid and the reversion frequency was determined using a tetracycline sensitivity assay. As shown in Table 4, the most mutagenic target is the +1 frameshift in both the mutS and wild-type strains with a reversion frequency 70-fold higher in the mutS strain, in agreement with its hypermutable background. Reversion in cells transformed with the −1 variant was not detected in either the mutS or the wild-type strains, and the +2 frameshift target was not reverted in the
wild-type and showed a low reversion frequency in the mutator strain.

DISCUSSION

In this work we describe the spontaneous emergence of new *P. aeruginosa* morphotypes upon inactivation of the MMRS by disruption of the mutS gene. Compared with the wild-type strain, the *P. aeruginosa* Hex1T mutS morphotypic variants in turn displayed differences in several phenotypic traits, including antibiotic resistance, altered motility behaviour, increased production of pyocyanin and pyoverdin, reduced LasB protease activity and a non-cytotoxic invasive phenotype. In spite of this diversification, which should be attributed to a higher mutation frequency, we observed that the extent of growth of the mutS strain was not altered compared to the wild-type and the analysis of chromosomal DNA by pulsed-field gel electrophoresis revealed no obvious genetic rearrangements (unpublished data).

The morphological diversification generated by the *P. aeruginosa* mutS strain is to our knowledge an original and striking observation. To deal with difficult environmental conditions, such as energy or oxygen limitations, motile bacterial colonies frequently diversify into a wide spectrum of morphological variants (Ben-Jacob et al., 1998). This morphological variation is thought to correlate with environmental heterogeneity, since adaptive morphological radiation of *Pseudomonas fluorescens* and *P. aeruginosa* populations has been observed in a heterogeneous environment provided either by static broth cultures or by biofilm growth (Déziel et al., 2001; Rainey & Travisano, 1998). Furthermore, *P. aeruginosa* isolated from CF patients displayed a great spectra of colony morphologies, probably as a response to the particularly high heterogeneity of the CF lung (Govan & Deretic, 1996; Häußler et al., 1999; 2003; Drenkard & Ausubel, 2002). In our assays, when *P. aeruginosa* Hex1T mutS was grown for a relatively short period, it reproducibly generated at least two morphotypes, mS1 and mS2, whereas in the same culture conditions such diversification was not observed in the wild-type strain. Again, it should be remarked that the growth rate of these mutS morphotypic variants not only did not show significant differences compared to the wild-type, but also they appear to have an improved growth advantage over wild-type in late stationary phase. Moreover, once isolated we observed that, in a reproducible and consistent way, one of the mutS morphotypes (mS2) emerged from the other mutS morphotype (mS1), suggesting the existence of a high-frequency pathway that produces such morphotypic diversification. Since at least under the conditions tested we did not observe a reversible phenomenon, the appearance of the mutS morphotypes does not seem to be due to a variation between two phases. However, it is important to point out that by performing an analysis of *P. aeruginosa* mS2 morphotypic variant growth in continuous-flow culture chambers, a new spectrum of mutS morphotypic variants was obtained from the biofilm effluents, including small adherent variants with impaired twitching motility (unpublished data). These observations suggest that environmental conditions are crucial to drive the generation and/or selection of these phenotypes, thus illustrating increased versatility in *P. aeruginosa* MMRS-deficient cells. As previously mentioned, the mutS morphotypic variants demonstrate significant differences in other phenotypical traits known to be regulated by quorum sensing, such as increased production of pigments, slightly increased rhamnolipid production, altered motility behaviour and reduced LasB protease activity. These observations support the idea that, even when it is likely that a variety of different mutations can generate the described phenotypes, the novel mutS morphotypic innovations may stem from mutations in a major regulator that simultaneously controls the expression of multiple phenotypic determinants and would be probably related to quorum sensing.

At the molecular level we postulate that frameshift mutations are important in the generation of these phenotypes. By using a tetracycline resistance gene reversion assay, we observed that mainly a +1 frameshift mutation was reverted, conferring antibiotic resistance, and that the

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>tet(^r) mutation</th>
<th>Position of tet(^r) mutation</th>
<th>No. of tet(^r) revertant cells per 10(^9) cells</th>
<th>mutS/wild-type revertants</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBBR1-W17</td>
<td>+ 1G</td>
<td>536</td>
<td>107 ± 63</td>
<td>7430 ± 650</td>
</tr>
<tr>
<td>pBBR1-W18</td>
<td>− 1G</td>
<td>536</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pBBR1-X2</td>
<td>+ GC</td>
<td>435</td>
<td>ND</td>
<td>0-9 ± 0-3</td>
</tr>
</tbody>
</table>

*Plasmids containing different frameshift mutations in the tetracycline resistance gene.

†The nucleotide position is indicated with respect to the initiation codon of the tetracycline gene.

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Table 4. Tetracycline reversion frequency of *P. aeruginosa* mutS and wild-type strains transformed with pBBR1-W17, pBBR1-W18 and pBBR1-X2

All determinations were carried out using approximately 10\(^9\) cells. Data are means ± SD of three experiments, each run in triplicate. ND, Not detected.
reversion frequency was 70-fold higher in the P. aeruginosa mutS strain. Nucleotide sequence analyses of the regions containing the frameshift mutations in several mutS and wild-type independent tetracycline revertant colonies confirmed that all the reversions analysed had a −1 deletion in the six-G repeat. This observation suggests that, as observed in E. coli and other bacterial species (Gur-Arie et al., 2000; Levy & Cebula, 2001), short G−C repeats are prone to slippage and constitute mutation hot-spots in the P. aeruginosa genome, particularly in a mutator strain.

In spite of the morphotypical diversification and in accordance with the hypermutator phenotype, both mutSmS1 and mS2 variants showed a high antibiotic resistant frequency when tested with several antibiotics commonly used in CF therapy. This observation is in concordance with previous studies that describe a significantly higher frequency of antibiotic-resistant cells in mutator P. aeruginosa pathogenic isolates than in non-mutator isolates, both obtained from CF patients, indicating a strong relationship between mutator phenotypes and antibiotic resistance (Oliver et al., 2000; Martinez & Baquero, 2000, 2002; Miller et al., 2002). Similar observations were obtained from mutator E. coli uropathogenic isolates (Denamur et al., 2002) as well as from in vitro (Mao et al., 1997) and in vivo (Giraud et al., 2002) experiments carried out with E. coli that have demonstrated that antibiotic treatment contributes to the selection of mutator cells. An interesting observation of our work was that antibiotic-resistant P. aeruginosa mutS clones are capable of increasing their resistance to high doses of antibiotic. This observation suggests that the mutator strain also generates a variable genetic background for the selection of highly antibiotic-resistant mutants probably by the accumulation of compensatory mutations (Bjorkman et al., 2000; Giraud et al., 2002). In addition, our results present further evidence to support the presumption that, for a pathogen, hypermutability is advantageous to overcome the effect of antibiotics and that antibiotic therapy could be one of the causes for selection of P. aeruginosa mutators in the CF lung (Oliver et al., 2000).

Another interesting observation was that whereas the wild-type strain displayed cytotoxic behaviour, variants that had lost their cytotoxicity and acquired an invasive capability were observed in both mS1 and mS2 populations, indicating that this phenotypic change is not related to morphotypical diversification and would occur at a high frequency. It is well known that P. aeruginosa can be internalized by (invasive) or can kill (cytotoxic) a variety of epithelial cells, including MDCK cells, corneal cells and human respiratory epithelial cells (Apodaca et al., 1995; Fleischig et al., 1994, 1996; Plotkowski et al., 1994). In our study the high frequency of invasive mutS cells observed was probably generated by the inactivation of genes involved in cytotoxicity and/or in anti-internalization functions. By scanning the genome sequence of P. aeruginosa we observed that several genes implicated in cytotoxicity and the type III secretion system have tracts of simple G−C repeats in their coding region (unpublished data). As observed in the tetracycline reversion assay, they would constitute hot-spots for frameshift mutations and may explain the high frequency loss of cytotoxicity in the mutS strain.

It is well known that P. aeruginosa virulence is multifaceted, even displaying differential pathologies. Furthermore, a wide phenotypic variation has been observed in P. aeruginosa clinical isolates (Govan & Deretic, 1996; Häußler et al., 2003), despite the fact that chronically infected CF patients harbour only one or a few genotypes (Breitenstein et al., 1997) and that there seems to be no genetic or functional differences between environmental and clinical strains (Alonso et al., 1999). These observations suggest that in P. aeruginosa, phenotypic diversification would constitute a relevant survival strategy associated with pathogenesis. Interestingly, we observed that the mutS strain generated morphotypic variants which in turn also diversify in several phenotypic traits mainly related to virulence, supporting the idea that MMRS-deficient cells have an important role in pathogenesis by favouring adaptive diversification. By the same token, according to our results and several previous reports we think that hypermutability could accelerate phenotypic variation switching mechanisms, such as phase variation and pathoadaptive mutations in P. aeruginosa (Richardson & Stojiljkovic, 2001; Sokurenko et al., 1998, 1999; Bucci et al., 1999).

It has been previously postulated that mutator cells would have an initial advantage in such environments in which new phenotypes are being continually selected for (Taddei et al., 1997; Funchain et al., 2000). However, it has also been discussed that this advantage is not conferred by hypermutability itself, but because of the association with rare favourable mutations (Taddei et al., 1997; Oliver et al., 2002). In this context, since morphotypical variability was also described in clinical isolates, it is possible to postulate that the in vitro morphotypical diversification capability of the mutS strain would exist in vivo and could constitute one of the bases of the high frequency of hypermutator P. aeruginosa in CF isolates (Oliver et al., 2000).

Although the molecular basis involved in the emergence of the observed morphotypes remains to be elucidated, this work provides useful information that will assist in the comprehension of the hypermutability effects on a P. aeruginosa population.

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