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9. Radiation hybrid (RH) mapping was performed with the IMpRH panel (*29*) and the RHMAP 3.0 software (*30*).
10. Sequence contigs constructed with PHRAP (*31*–*33*) were masked for repeats using RepeatMasker, available online at http://ftp.genome.washington.edu/cgi-bin/RepeatMasker.
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38. Muscle tissue was collected from four rn^+ (rn^+/rn^+) and five RN^- (two RN^-/rn^+ and three RN^-/RN^-) pigs. Muscle extract was purified up to and including the DEAE-Sepharose ion-exchange step as described (*35*). AMPK activity was assayed by phosphorylation of a synthetic peptide, HMRSAMSGLHLVKRR (*36*, *37*). Assays were performed in triplicate for each animal in the presence and in the absence of 200 μM AMP. Because 0.1 μg of total protein from the 0.2 M NaCl DEAE eluates showed a linear activity both in the presence and absence of added AMP for at least 12 min (D. Milan *et al.*, data not shown), we used this amount of extract and an incubation time of 10 min in the experiments. For each assay, we included controls that lacked added peptide. The values presented represent peptide-dependent activity.
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High Frequency of Hypermutable *Pseudomonas aeruginosa* in Cystic Fibrosis Lung Infection

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The lungs of cystic fibrosis (CF) patients are chronically infected for years by one or a few lineages of *Pseudomonas aeruginosa*. These bacterial populations adapt to the highly compartmentalized and anatomically deteriorating lung environment of CF patients, as well as to the challenges of the immune defenses and antibiotic therapy. These selective conditions are precisely those that recent theoretical studies predict for the evolution of mechanisms that augment the rate of variation. Determination of spontaneous mutation rates in 128 *P. aeruginosa* isolates from 30 CF patients revealed that 36% of the patients were colonized by a hypermutable (mutator) strain that persisted for years in most patients. Mutator strains were not found in 75 non-CF patients acutely infected with *P. aeruginosa*. This investigation also reveals a link between high mutation rates in vivo and the evolution of antibiotic resistance.

Cystic fibrosis is a human genetic disorder caused by mutations in the CF-transmembrane conductance regulator (*1*). Mutations in the gene encoding this protein disrupt electrolyte secretion, leading to a hyperosmolar viscous mucus (*2*). The main mechanisms of lung defense against bacterial colonization are mucociliary clearance, polymorphonuclear neutrophil phagocytosis, and local production of antibacterial cationic peptides. These systems of defense are poorly effective under conditions of increased viscosity and osmolarity, resulting in chronic lung infection, most frequently by *Pseudomonas aeruginosa*, the major cause of morbidity and mortality in CF patients (*3*, *4*).

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Although the lungs of CF patients are rich in organic compounds for bacterial growth, bacteria must continually evolve to adapt to limitations in specific growth factors, dehydration, leukocyte influx, the physical (ecological) heterogeneity of the deteriorating lung tissue, and frequently changing and prolonged (over a period of years) antibiotic therapy.

Despite its underlying clonal structure, there is a significant variation in the phenotypes of *P. aeruginosa* isolated from CF patients (*5*). In contrast to the monomorphic colony types of *P. aeruginosa* isolated from acute clinical infections, such as those obtained from the blood of septicemic patients, the isolates from CF patients display a wide spectrum of colony variants, including mucoid and dwarf colonies. Moreover, *P. aeruginosa* isolated from CF patients include otherwise isogenic variants that are nonmotile, nonflagellated, lipopolysaccharide-deficient, auxotrophic, or resistant to antibiotics (*6–8*). Furthermore, the development of the *P. aeruginosa* mucoid phenotype, which is a key step in the establishment of the chronic lung infection, usually involves the acquisition of stable mutations (*9*).

Recent theoretical and laboratory studies (*10–12*) suggest that the adaptation of bacteria to a heterogeneous and changing environment would promote the selection of hypermutable (mutator) strains. In vitro, hypermutable strains are produced mainly by alterations in the DNA repair and error-avoidance genes (*13*). Accordingly, *P. aeruginosa* isolated from CF patients, unlike those obtained from patients with acute infections, should have a high frequency of mutator strains. To test this hypothesis, we estimated the mutation frequencies of *P. aeruginosa* from CF patients and from patients with acute *P. aeruginosa* infections.

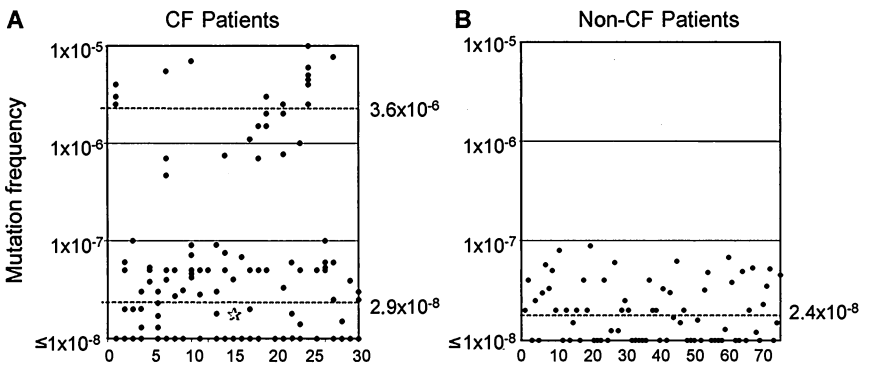

Fig. 1. Rifampicin mutation frequencies for 128 CF *Pseudomonas aeruginosa* isolates grouped by patients (**A**) and 75 isolates from non-CF patients (**B**). Dashed lines represent the mean of mutator and nonmutator groups; the PAO1 control strain (for which the genome sequence is known) is represented as ☆. One hundred and forty *P. aeruginosa* isolates were randomly selected from sputum samples obtained during 1993 to 1998 from 30 chronically infected CF patients. Only patients with at least 3 years of documented *P. aeruginosa* colonization were included (mean age of 22 years). Because a single patient may be colonized by different strains, a minimum of two and a maximum of six isolates per patient were studied. To reduce duplication of the collected organisms, one isolate per year was randomly selected from a given patient. Serotype, phagetype, colonial morphotype, and antibiotic-resistance pattern were used to discriminate between identical isolates from the same patient. In case of identity, only one isolate was retained and used in the study. Based on these criteria, 12 isolates (including 2 mutators) were excluded. As a control group, a collection of 50 randomly obtained *P. aeruginosa* isolates from blood cultures, and 25 obtained from respiratory samples in different and epidemiologically unrelated non-CF patients, during the same period, were included. The *P. aeruginosa* standard laboratory strain PAO1 was included as control.

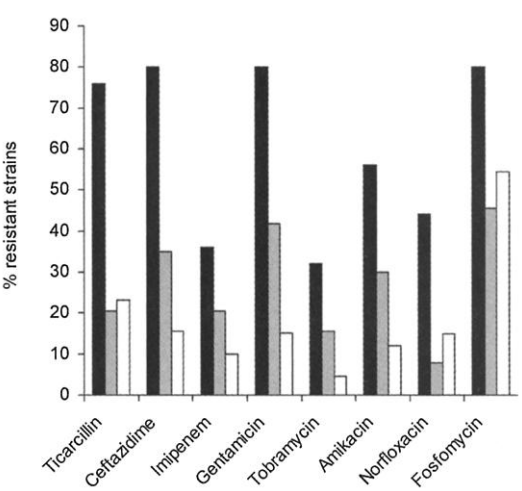

Fig. 2. Differences in antibiotic resistance in *P. aeruginosa* between mutator CF isolates (black bars), nonmutator CF isolates (gray bars), and non-CF isolates (white bars). Statistically significant differences between mutator and nonmutator CF isolates (Fisher's test) were found for ticarcillin ($P < 0.001$), ceftazidime ($P < 0.001$), gentamicin ($P < 0.001$), amikacin ($P = 0.015$), norfloxacin ($P = 0.0053$), and fosfomycin ($P = 0.0017$). Differences did not reach statistical significance for imipenem ($P = 0.085$) and tobramycin ($P = 0.058$).

Of the 128 *P. aeruginosa* isolates obtained from the sputum of 30 CF patients, 19.5% exhibited a mutator phenotype (*15*). Mutator strains were obtained from 11 patients (36.7%). In these patients, the overall proportion of isolates with a mutator phenotype versus a nonmutator phenotype was 43.1%. No *P. aeruginosa* isolate from 50 blood and 25 respiratory samples from 75 non-CF patients exhibited the mutator phenotype. The mutation frequency distributions are shown (Fig. 1). Two groups of isolates from CF patients were distinguished: a group of nonmutators, with a mean mutation frequency of $2.9 \pm 2.5 \times 10^{-8}$, and a group of mutators, with a mean mutation frequency of $3.2 \pm 2.5 \times 10^{-6}$ (Fig. 1A). All isolates recovered from blood and respiratory samples from non-CF patients had a low mutation frequency (mean: $2.4 \pm 2.1 \times 10^{-8}$) (Fig. 1B).

To explore the genetic structure (i.e., the extent of clonality) and the epidemiology of the *P. aeruginosa* population within and between CF patients, we performed random amplification of polymorphic DNA (RAPD) assays on chromosomal DNA from the 128 CF isolates (*15*). These results suggested that the *P. aeruginosa* flora of these patients was dominated by a single strain or very few lineages that persisted over many years. Twenty-five CF patients harbored a single RAPD type, three CF patients harbored two RAPD types, and two CF patients harbored three RAPD types. There was no evidence of interpatient transmission of these types. Every patient harboring mutator isolates had a different RAPD-type strain, and in most cases, the type strain was consistently recovered over the years. This observation supports the interpretation that the mutators had evolved within these patients.

The presence of major genetic changes in the mutator genes of the corresponding mutator strains of *P. aeruginosa* was explored by polymerase chain reaction (PCR) analysis (*16*). Two isolates from the same patient, obtained within a 4-year interval, had an identical ~1.5-kb deletion in the *mutS* gene region. Four isolates failed to amplify the *mutY* gene (three isolates from the same patient, obtained in three consecutive years, and one from a different patient) (*17*). Furthermore, the increased mutation frequency of the mutator strains from 4 of the 11 patients (including the isolates with a deletion in *mutS*) was complemented with the cloned PAO1 *mutS* gene (*18*). Point mutations in these or other mutator genes may be responsible for the high mutation frequency found in the other mutator strains (*19*).

The differential activity of antibiotics on mutator and nonmutator lung isolates and non-CF blood and lung isolates is shown in Fig. 2. Minimal inhibitory concentrations were deter-

mined according to the NCCLS recommended criteria (*20*). There is a significant difference in resistance to several antibiotics, being roughly double in frequency for mutator isolates. This result may explain the higher resistance rates commonly found in *P. aeruginosa* isolates from CF lungs compared with non-CF isolates. In *P. aeruginosa* point mutations confer resistance to β-lactams, aminoglycosides, quinolones, and fosfomycin. All CF patients received several cycles of combinations of these antimicrobial agents previously and during the study. High rates of mutation may be of benefit for the production and fixation of some antibiotic-resistance mutations having a fitness cost for the organism, because there is also a higher possibility of cost-compensatory mutations (*21*).

The compartmentalized nature of the bronchial habitat in CF patients also means that local antibiotic concentrations will vary, and this will enhance the evolution of drug resistance (*22*, *23*) in mutant-specific, antibiotic-selective compartments (*24*). Compartmentalized habitats ensure small local population sizes, which would be less subject to "speed limits" [i.e., when a further increase of mutation rate will not favor population fitness owing to clonal interference (*25*)]. Severe population bottlenecks would be expected to occur after strong selective challenge, such as antibiotic pressure, thus potentially giving any consequently selected mutators a significant role in bacterial evolution (*26*).

The evolutionary ecology of *P. aeruginosa* in the lungs of CF patients is an example of adaptive radiation. Adaptive radiation of genetically uniform *Pseudomonas* populations into a variety of colonial morphotypes has recently been demonstrated experimentally in in vitro compartmentalized habitats (*27*). The progressive anatomical deterioration of the CF lung during chronic infection also provides a highly spatially structured environment, and indeed, diversification into new morphological types is a typical feature of *P. aeruginosa* chronic bacterial infection (*28*).

Bacterial populations undergoing long-term adaptation to new or challenging environments spontaneously generate mutators (*11*, *12*). An earlier in vitro experiment has shown that mutator *Escherichia coli* cells can proliferate in a population from $<10^{-5}$ to 2.5×10^{-1} after two sequential selection steps (*29*). In natural *E. coli* isolates, high mutation rates are caused by mutations in *mutH*, *mutL*, *mutS*, *uvrD*, or *mutT* genes (*30*, *31*). The incidence of mutator strains was found to be higher (over 1%) among isolates of pathogenic *E. coli* and *Salmonella enterica* than among nonpathogenic ones (*32*). Although the unexpected high frequency of mutators in this work suggested a link between the mutator phenotype and pathogenicity, this relation was later challenged when a similar frequency of mutator strains was found among commensal and pathogenic *E. coli* strains in humans (*31*).

The high proportion of mutators in isolates from CF patients suggests that rapid adaptation is required by bacterial populations to survive in the lungs of these patients. Once adapted, the mutator is expected to revert to the original nonmutator state (*10*) because deleterious mutations may accumulate in mutator populations and decrease their fitness. Nevertheless, reversion to nonmutators seemed to be infrequent in the *P. aeruginosa* CF lung isolates because the same mutator strains were consistently recovered for years in most patients. The accumulation of adaptive mutations has been suggested as an explanation for the fixation of the mutator genotype in abundant populations (*10*, *34*). Moreover, the changing characteristics of the CF lung environment ensures the perpetuation of mutators because the selection pressure on *P. aeruginosa* is never lifted. Fixation of antibiotic resistant mutators, as seen in our work, certainly creates a therapeutic challenge because the mutators are not only more resistant to antibiotics but are also more likely to become resistant to new compounds. Thus, the combination of high total cell density, a multiplicity of environmental challenges, and a changing and compartmentalized habitat create a unique selective scenario for bacterial mutator populations. This study offers evidence for the impact of mutator phenotypes in a clinical setting and shows a relation between mutator phenotypes and high antibiotic resistance in *P. aeruginosa* isolates from CF patients.

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17. DNA from these four isolates was checked by amplifying in the same PCR tube another gene with higher molecular weight (*uvrD*) that, in contrast to *mutY*, was correctly amplified.
18. The *mutS* gene from the control strain PAO1 was cloned and sequenced (GenBank accession number AF220055). The *Pseudomonas pJMS6αlac* plasmid harboring the *mutS* gene was introduced by conjugation into mutator strains from the 11 patients with mutators. It was also introduced in 10 nonmutator strains from CF patients and in 10 nonmutator strains from blood or respiratory tract from non-CF patients. The same plasmid lacking the *mutS* gene was introduced in all strains as a control. All experiments were done in three independent transconjugant colonies. Reversion ($>10^2$ decrease in mutation frequency) was found in mutator strains from 4 out of the 11 CF patients with mutators. No significant change in mutation frequency was detectable in non-mutator strains or with the plasmid lacking the *mutS* gene.
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