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Hypermutation Is a Key Factor in Development of Multiple-Antimicrobial Resistance in *Pseudomonas aeruginosa* Strains Causing Chronic Lung Infections

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***Pseudomonas aeruginosa* is the most relevant pathogen producing chronic lung infections in patients with chronic underlying diseases such as cystic fibrosis (CF), bronchiectasis, and chronic obstructive pulmonary disease (COPD). Hypermutable (or mutator) *P. aeruginosa* strains, characterized by increased (up to 1,000-fold) spontaneous mutation rates due to alterations of the DNA mismatch repair (MMR) system have been found at high frequencies in the lungs of CF patients, but their role in other chronic processes is still unknown. Sixty-two *P. aeruginosa* isolates from 30 patients with underlying non-CF chronic respiratory diseases (22 with bronchiectasis and 8 with COPD) and documented chronic infection were studied. Antibiotic susceptibility profiles and mutation frequencies were determined, and complementation assays using the cloned wild-type *mutS* gene and molecular epidemiology studies (pulsed-field electrophoresis, [PFGE]) were performed with these strains. Thirty-three (53%) of the isolates were hypermutable, and 17 (57%) of the 30 patients were colonized by hypermutable strains. Strains from 11 of the 17 patients were found to be defective in the MMR *mutS* gene by complementation assays. Interpatient transmission of strains was ruled out by PFGE. Multiple-antimicrobial resistance was documented in 42% of the hypermutable strains in contrast to 0% resistance in the nonhypermutable strains ($P < 0.0001$). Hypermutable *P. aeruginosa* strains are extremely prevalent in chronic infections in contrast to what has been described in acute processes, suggesting a role of hypermutation in bacterial adaptation for long-term persistence. Furthermore, hypermutation is found to be a key factor for the development of multiple-antimicrobial resistance, and therefore these findings are expected to have important consequences for the treatment of chronic infections.**

Pseudomonas aeruginosa is a ubiquitous versatile environmental microorganism that is the leading cause of opportunistic human infections (42). This pathogen is one of the major and more severe causes of acute nosocomial infections, especially affecting patients in intensive care units (ICUs) with mechanical ventilation-associated pneumonia or burn wound infections, which are both associated with a high mortality rate (22, 45). Nevertheless, *P. aeruginosa* is also a major cause of chronic respiratory infections. For instance, this microorganism is the main driver for morbidity and mortality of cystic fibrosis (CF) patients (7, 9, 21); is the leading cause of chronic infection in patients with bronchiectasis, which is associated with more severe lung function deterioration and poorer quality of life (5, 26, 28, 46); and is beginning to be recognized as a marker of intense airway inflammation in patients with chronic obstructive pulmonary disease (COPD) (13). The prevalence of *P. aeruginosa* in patients with COPD is around 4% but increases to 8 to 13% in patients with advanced airflow obstruction (19).

One of the most striking characteristics of *P. aeruginosa* is its extraordinary ability for antibiotic resistance acquisition (20). Treatment failure due to the development of resistance is indeed a frequent outcome of *Pseudomonas* infections (6). This is an especially critical factor in the management of chronic infections such as those occurring in CF patients. After years of intensive antibiotic chemotherapy in an effort to control the negative outcome of chronic colonization, sequential development of resistance to most antibiotics frequently occurs. Resistance rates of *P. aeruginosa* strains isolated from CF patients are, in fact, substantially higher than those found in other settings, including the strains from patients in ICUs (12, 29). Considerably less information is available for other *P. aeruginosa* chronic infection settings, such as bronchiectasis or COPD. Nevertheless, a limited number of studies have demonstrated that *P. aeruginosa* strains isolated from CF and bronchiectasis patients share common features, such as the frequent emergence of mucoid isolates, the intense phenotypic diversification despite unique colonizing clonal lineages, and the frequent emergence over time of antibiotic-resistant variants (9, 11, 14, 33, 38).

A link between the high antibiotic resistance rates in CF patients and the presence of a high proportion of hypermutable (or mutator) *P. aeruginosa* strains has been previously documented (29). Mutators represented 20% of the total number of *P. aeruginosa* isolates from CF patients and were found in 37% of the patients in the mentioned study (29). Hypermut-

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able strains are those that have an increased (up to 1,000-fold) spontaneous mutation rate due to defects in genes involved in DNA repair or error avoidance systems (15, 25). In *P. aeruginosa* strains obtained from CF patients, as well as in other natural bacterial populations (18, 24), the most frequently involved system is the mismatch repair (MMR) system, and *mutS* is the most frequently affected gene (30). A recent in vitro work has shown that *mutS* deficiency in *P. aeruginosa* determines the immediate development of resistance to every single antipseudomonal agent due to the ascent to dominance of resistant mutants in a few hours during drug exposure (31).

In order to ascertain whether hypermutation is a common feature of chronic lung infections caused by *P. aeruginosa*, a prospective study on non-CF patients with underlying chronic respiratory diseases was undertaken, finding that the prevalence of hypermutable *P. aeruginosa* strains is extremely high (more than 50% of the isolates) in these patients. Furthermore, hypermutation is found to be the main driver for the development of multiple-antimicrobial resistance in patients with chronic infections caused by *P. aeruginosa*.

MATERIALS AND METHODS

Patients and strains. From March 2003 to June 2004, all *P. aeruginosa* isolates from respiratory samples submitted to the Microbiology Department from patients with documented underlying chronic respiratory diseases, excluding cystic fibrosis, were prospectively collected. Identification of the isolates was performed with the API 20NE system (Biomérieux, France) and conventional tests. For the definition of chronic *P. aeruginosa* colonization/infection, only patients with at least 1 year of documented *P. aeruginosa* colonization prior to the study (according to the Microbiology Department database, with information available up to 1995) or with at least three *P. aeruginosa*-positive cultures, each separated by more than 1 month, during the study period were considered. All different *P. aeruginosa* morphological variants isolated from two sputum samples (separated by more than 1 month) from each of the patients with documented chronic colonization were selected for further study. In those patients with only one sample recovered during the study period, all different *P. aeruginosa* morphological variants from these single samples were included. Strain PAO1 and its hypermutable, *mutS*-deficient, isogenic derivative, PAOΔ*mutS* (31), were used as controls.

According to a American Thoracic Society/European Respiratory Society consensus document (2), COPD was diagnosed as not fully reversible chronic obstruction to airflow demonstrated by forced postbronchodilator spirometry (forced expiratory volume in 1 s [FEV1]/forced vital capacity [FVC], <0.70) which could not be explained by other pulmonary pathology. The severity of the chronic obstruction to airflow was assessed using the Global Initiative for Chronic Obstructive Lung Disease criteria (2) that classify patients in four categories: mild (decreased FEV1/FVC with normal FEV1), moderate (FEV1 between 50 and 80% of the predicted value), severe (FEV1 between 30 and 50%), and very severe (FEV1 $<$ 30%).

Considering the criteria described by Kang et al. (16), bronchiectasis was diagnosed by computed tomography (CT) chest scan on the basis of an internal bronchial diameter that was greater than that of the adjacent pulmonary artery, lack of tapering of the bronchial lumina, and/or visualization of bronchi within 1 cm of the pleura. Other roentgenologic signs characteristic of bronchiectasis based on the CT classification criteria of Naidich et al. (27) were as follows: bronchial-wall widening or absence of tapering in contiguous axial sections for mild cylindric bronchiectasis, horizontal or vertical course of the bronchi seen as "tramlines" or "signet rings" for cylindric bronchiectasis, dilated bronchi with walls of beaded appearance for varicose bronchiectasis, and dilated bronchi with air fluid levels and/or strings or clusters of cysts for cystic bronchiectasis. A respiratory function test (spirometry) was performed for all patients with bronchiectasis in order to assess the presence of chronic obstruction to airflow and the severity of the obstruction. Obstruction in bronchiectasis was defined by a prebronchodilator FEV1/FVC value of <0.70, and the severity was assessed as for COPD.

Antibiotic susceptibility testing. MICs were determined for ceftazidime, imipenem, meropenem, ciprofloxacin, and tobramycin in Mueller-Hinton (MH) agar plates by using Etest strips (AB Biodisk, Sweden) following standard procedures, and the resistance breakpoints used were those of the Clinical and Laboratory Standards Institute (formerly NCCLS) document M100-S15 (4). The documentation and interpretation of the antibiotic-resistant mutant subpopulations for the detection and susceptibility testing of hypermutable strains were performed as previously described (23). Briefly, suspensions with a McFarland standard of 0.5 or 1 were used for inoculum standardization of regular or mucoid isolates, respectively. MICs were read after 24 h (36 h for slow-growing strains) of incubation at 37°C. The presence (or absence) of resistant mutant subpopulations within the inhibition zones, as well as their relative numbers (+, ++, and +++ for <10, 10 to 100, and >100 mutants, respectively) and their highest MICs, was recorded after an extra 12 h of incubation. Except when specifically indicated, the MICs considered for the analysis were those of the general bacterial population regardless of the presence of resistant mutant subpopulations.

Estimation of mutation frequencies and complementation studies. Mutation frequencies were estimated as previously described (29, 30). Briefly, independent triplicate 10-ml overnight MH broth cultures of the *P. aeruginosa* isolates were collected by centrifugation and resuspended in 1 ml of saline solution. Serial 10-fold dilutions were plated in MH agar with and without 300 μg/ml of rifampin, and after 36 h of incubation (48 h for slow-growing strains), colonies were counted and the mean fraction of mutants estimated. As previously defined, strains were considered hypermutable when the mutation frequency was at least 20-fold higher than that obtained for control strain PAO1 (28). To explore the genetic basis for the mutator phenotype, complementation studies using the cloned PAO1 wild-type *mutS* gene were performed with all hypermutable strains. Plasmid pUCPMS harboring the PAO1 *mutS* gene (31) was electroporated into the different hypermutable *P. aeruginosa* isolates as previously described (40). Transformant cells harboring plasmid pUCPMS were selected in Luria-Bertani agar plates containing 50 or 250 μg/ml gentamicin. Complementation of the increased mutation frequencies was studied in three independent transformants.

Molecular epidemiology. The epidemiological relatedness of the strains was studied by pulsed-field gel electrophoresis (PFGE). Bacterial DNA embedded in agarose plugs prepared as previously described (17) was digested with the restriction enzyme SpeI. DNA separation was performed in a CHEF-DRIII apparatus (Bio-Rad, La Jolla, Calif.) under the following conditions: 6 V/cm^2 for 26 h with pulse times of 5 to 40 seconds. DNA macrorestriction patterns were interpreted according to the criteria established by Tenover et al. (44)

Statistical analysis. Fisher's exact test and the Mann-Whitney U test were used for the comparison of resistance rates and distributions of MICs, respectively, between hypermutable and nonhypermutable strains. A P value of <0.05 was considered statistically significant.

RESULTS

A total of 45 patients with documented underlying chronic lung disease (26 with bronchiectasis and 19 with COPD) had at least one *P. aeruginosa*-positive sputum culture during the study period. Following the criteria described in Materials and Methods, 30 of them were considered to have documented chronic *P. aeruginosa* colonization. A total of 62 *P. aeruginosa* isolates from two sputum samples from each of these 30 patients were studied. Estimations of the mutation frequencies revealed that 33 (53%) of the isolates were hypermutable. The distribution of the mutation frequencies is shown in Fig. 1. The mean mutation frequency of nonhypermutable isolates was 1.8 mutants/10^8 cells (range, 0.07 to 7.7 mutants/10^8 cells), and that of hypermutable isolates was 470 mutants/10^8 cells (range, 52 to 2,100 mutants/10^8 cells). Seventeen (57%) of the 30 patients were found to be colonized by hypermutable strains. Hypermutable strains from 11 (65%) of the 17 patients were found to be defective in the MMR *mutS* gene by complementation assays using plasmid pUCPMS. The range of the mutation frequencies of *mutS*-deficient strains was similar to that of the overall hypermutable strains (from 56 to 1,200 mutants/10^8 cells).

To evaluate the possibility of the high prevalence of hypermutable strains being a consequence of the dissemination of a single clone (or a few) of hypermutable *P. aeruginosa*, molecular epidemiology studies were undertaken. The analysis of the

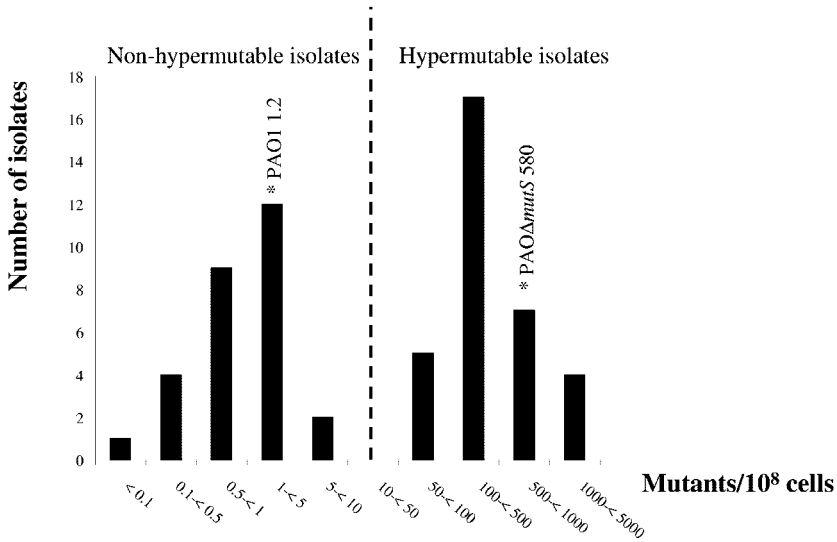

FIG. 1. Distribution of mutation frequencies of the 62 *P. aeruginosa* isolates from chronically infected patients. Mutation frequencies of the control strain PAO1 and its hypermutable derivative PAOΔ*mutS* are also shown.

PFGE DNA macrorestriction patterns from the collection of 62 *P. aeruginosa* isolates revealed no interpatient transmission of strains. A total of 32 different *P. aeruginosa* clones were identified, each found in a single patient. In 28 of the 30 patients, a single *P. aeruginosa* clone was documented, whereas the remaining 2 patients were found to harbor two different clones. When two or more *P. aeruginosa* morphological variants were recovered from a single sample, which occurred for 10 of the 30 patients, analysis of PFGE patterns of the different isolates revealed the presence of a single clonal type as is frequently observed in CF patients (38). Twenty-eight (45%) and 13 (21%) of the isolates had the mucoid- or small-colony variant morphotypes, respectively, typical of chronic CF infection (9, 11). Nevertheless, no significant differences in the proportions of these morphotypes between hypermutable and nonhypermutable isolates were observed.

To evaluate the impact of the high prevalence of hypermutable strains on antibiotic resistance, susceptibility testing for five of the most potent and widely used antipseudomonal agents was performed. Overall antibiotic resistance percentages for the 62 isolates ranged from 3.2% for meropenem to 35.5% for ciprofloxacin (Table 1). When resistance percentages of hypermutable and nonhypermutable strains were analyzed independently, it was found not only that hypermutable strains were much more resistant to all the antibiotics but also that most of the strains resistant to any of the antibiotics were hypermutable. The differences in resistance percentages were statistically significant (Fisher's exact test) for ceftazidime (P = 0.0056), imipenem (P = 0.0019), and ciprofloxacin (P = 0.0048). Differences did not reach statistical significance for meropenem and tobramycin due to the low number of strains resistant to these antibiotics. However, when the MIC distributions were analyzed for these two antibiotics, hypermutable strains were found to be significantly (Mann-Whitney U test)

TABLE 1. Percentages of resistance and geometric means of MICs for *P. aeruginosa* isolates with and without considering resistant mutant subpopulations

Antibiotic	Overall isolates (n = 62)		Nonhypermutable isolates (n = 29)		Hypermutable isolates (n = 33)		Statistical significance (P)[c]	Hypermutable isolates considering RMS[d] (n = 33)		Overall % resistance considering RMS of hypermutable isolates[e]
	No. (%) resistant[a]	Mean MIC[b]	No. (%) resistant	Mean MIC	No. (%) resistant	Mean MIC		No. (%) resistant	Mean MIC	
Ceftazidime	11 (17.7)	1.5	1 (3.5)	0.68	10 (30.3)	3.6	0.0056	23 (69.7)	37.9	38.7
Imipenem	9 (14.5)	1.1	0 (0)	0.53	9 (27.3)	1.9	0.0019	25 (75.8)	8.5	40.3
Meropenem	2 (3.2)	0.09	0 (0)	0.04	2 (6.1)	0.18	0.27	9 (27.3)	1.4	14.5
Ciprofloxacin	22 (35.5)	0.65	5 (17.2)	0.31	17 (51.5)	1.2	0.0048	27 (81.8)	5.5	51.6
Tobramycin	6 (9.7)	1.2	1 (3.5)	0.90	5 (15.2)	1.7	0.13	11 (33.3)	2.7	19.3
≥2 antibiotics	14 (22.6)		0 (0)		14 (42.4)		<0.0001	33 (100)		53.2

[a] The numbers and percentages of resistant isolates are according to the Clinical and Laboratory Standards Institute non-susceptibility breakpoints and therefore include the intermediate and resistant categories.
[b] Geometric mean of the MICs in μg/ml.
[c] P values (Fisher's exact test) resulting from comparison of the numbers of resistant strains among nonhypermutable and hypermutable isolates.
[d] RMS, resistant mutant subpopulations. Results are based on the MICs of the resistant mutant colonies growing within the inhibition zones.
[e] Overall percentages of resistance obtained for the 62 studied *P. aeruginosa* isolates if the RMS of hypermutable strains were considered in the susceptibility results interpretation.

more resistant (higher MICs) than nonhypermutable strains both for meropenem ($P = 0.003$) and for tobramycin ($P = 0.021$). When multiple-antimicrobial resistance was considered, an even higher impact of hypermutation was observed. Forty-two percent (14 out of 33) of the hypermutable strains were resistant to at least two of the antibiotics tested, whereas none of the 29 nonhypermutable strains were found to be resistant to more than one antimicrobial agent ($P < 0.0001$); in other words, all *P. aeruginosa* strains resistant to two or more antibiotics (22.6%) were hypermutable.

Hypermutable strains are characterized by the presence of resistant mutant subpopulations within the inhibition zones of the antibiotics due to the high spontaneous mutation rates (23). When these typically resistant mutant subpopulations were considered in susceptibility data interpretation, the rates of resistance for the hypermutable strains were increased much further, reaching values ranging from 27% for meropenem to 82% for ciprofloxacin (Table 1). Then, given the high proportion of hypermutable strains, the consideration of the resistant mutant subpopulations had an important additional effect on the overall resistance rates, as can be observed in Table 1.

DISCUSSION

Pseudomonas aeruginosa is the most relevant pathogen producing chronic lung infections in patients with chronic underlying diseases such as CF and bronchiectasis and is beginning to be recognized as a relevant pathogen in COPD patients with advanced airflow obstruction. While long-term treatment with antipseudomonal agents is crucial to avoid a fast decline in the respiratory function of the infected patients, mutants resistant to multiple antimicrobials almost invariably evolve and frequently lead to treatment failure. Unlike many other bacteria, *P. aeruginosa* can, by changes in single genes, generate mutants that are resistant to clinical concentrations of all antimicrobial agents used for therapy (20).

In vitro and theoretical approaches suggest that the acquisition of a stable mutator phenotype may confer a selective advantage for bacteria, particularly in stressful and fluctuating environments (41, 43). In vivo experiments have also shown a potential benefit of hypermutation for bacterial adaptation to new environments, although once adapted, this advantage tends to disappear, and the transmissibility of the mutator strains is considerably reduced (8). In natural bacterial populations, the presence of hypermutable strains has been linked to resistance to host immunological defenses, such as mutation-dependent phase variation in *Neisseria meningitidis* (36) or resistance to antibiotics in *P. aeruginosa* isolates from chronically infected CF patients (29). Up to 37% of the CF patients were colonized by hypermutable strains in the mentioned study (29), whereas not a single hypermutable strain was found in 75 acutely infected patients. Recently, results from another study have suggested that chronicity itself maybe a key factor for the selection of hypermutable *P. aeruginosa* strains: despite the fact that mutational-antibiotic resistance development was a frequent outcome in a series of 103 acutely infected ICU patients, the prevalence of hypermutable strains was found to be lower than 1% (10). Certainly, the extremely high prevalence of hypermutable strains found in the present work suggests that hypermutation plays a role in the bacterial adaptation to the lung environment required for long-term persistence because hypermutation increases the rates of generation of any mutant phenotype (not only antibiotic resistance), including those involved in the development of resistance to the host immune system. In this sense, it has recently been found in vitro that mismatch repair disruption in *P. aeruginosa* determines the emergence at a high frequency of multiple morphological variants resembling what naturally occurs in chronic infections (39). Furthermore, the link between hypermutation and chronic infections seems not to be restricted to just *P. aeruginosa*, since recent works have found a significantly higher prevalence (14%) of hypermutable *Staphylococcus aureus* and *Haemophilus influenzae* strains in CF patients than in patients with other infections (1%) (32, 37).

Whether the impressive high frequency of hypermutable *P. aeruginosa* strains that we have found in chronic infections is a consequence of (i) coselection of the hypermutable variants with the adaptive mutations required for long-term bacterial persistence, (ii) their coselection with antibiotic resistance mutations required for survival during the frequent antibiotic treatments received by these patients, or, more likely, (iii) both selecting forces acting synergistically remains to be elucidated. Nevertheless, results from this work strongly suggest that hypermutation is the main driver for the development of antibiotic resistance in chronic *P. aeruginosa* infections, since most of the strains resistant to any of the antibiotics and all of the strains resistant to multiple antibiotics were hypermutable.

In the last 2 years, the role of hypermutation in antibiotic resistance development has begun to be acknowledged as a potentially concerning problem (1, 3). A recent in vitro work has shown that *mutS* deficiency in *P. aeruginosa* determines the immediate development of resistance to every single antipseudomonal agent, due to the ascent to dominance of resistant mutants in a few hours during drug exposure, and therefore monotherapy should be avoided in the treatment of infections by hypermutable strains (31). These in vitro results, together with those obtained in the present study, certainly demonstrate that hypermutation is a major negative factor for the treatment of chronic infections not only because hypermutable strains are more frequently resistant to the antibiotics, limiting our therapeutic arsenal, but also because development of resistance to additional antibiotics is expected to be extremely fast during therapy.

Early treatment of *P. aeruginosa* lung colonization in patients with underlying chronic respiratory diseases before the typical markers of chronic *P. aeruginosa* infection, among them hypermutation, are selected should be the main therapeutic strategy (35). Once the chronic infection is established and the hypermutable strains selected, eradication is expected to be almost impossible, and avoidance of treatment failure due to multiple-antimicrobial resistance development should be the main objective. Maintenance inhaled-tobramycin treatment has been found to have a positive effect on the pulmonary function of CF patients, whereas resistance selection has been found to be relatively low due to the higher (up to 100-fold) antibiotic concentrations reached in the colonization site than by the systemic route (34). The high antibiotic concentrations may prevent resistance development even in the hypermutable strains, since the accumulation of multiple resistance mutations is required for reaching such high-level resistance.

In summary, in this work we find that hypermutable *P. aeruginosa* strains are extremely frequent in chronic lung infections, in contrast to what is observed in acute processes. Furthermore, hypermutation is found to be the main driver for the development of multiple-antimicrobial resistance in patients with chronic *P. aeruginosa* infections. In order to avoid this negative outcome, treatment strategies should be directed to the avoidance of the selection of hypermutable variants.

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