

## Occurrence of Hypermutable *Pseudomonas aeruginosa* in Cystic Fibrosis Patients Is Associated with the Oxidative Stress Caused by Chronic Lung Inflammation

Oana Ciofu,<sup>1\*</sup> Bente Riis,<sup>2</sup> Tacjana Pressler,<sup>3</sup> Henrik Enghusen Poulsen,<sup>2</sup>  
and Niels Høiby<sup>1,4</sup>

*Institute for Medical Microbiology and Immunology, Panum Institute, University of Copenhagen, Copenhagen, Denmark*<sup>1</sup>; *Institute of Clinical Pharmacology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark*<sup>2</sup>; *Cystic Fibrosis Center, Pediatric Department, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark*<sup>3</sup>; and *Department of Clinical Microbiology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark*<sup>4</sup>

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**Oxidative stress caused by chronic lung inflammation in patients with cystic fibrosis (CF) and chronic lung infection with *Pseudomonas aeruginosa* is characterized by the reactive oxygen species (ROS) liberated by polymorphonuclear leukocytes (PMNs). We formulated the hypothesis that oxidation of the bacterial DNA by ROS presents an increased risk for the occurrence of hypermutable *P. aeruginosa*. The occurrence of hypermutable *P. aeruginosa* isolates was investigated directly in the sputum of 79 CF patients and among 141 isolates collected from 11 CF patients (10 to 15 isolates/patient) collected from the 1st and up to the 25th year of their chronic lung infection. The level of oxidized guanine moiety 8-oxo-2'-deoxyguanosine (8-oxodG), which is a frequently investigated DNA oxidative lesion, was measured. Hypermutable *P. aeruginosa* isolates were found in the sputum bacterial population of 54.4% of the CF patients. The earliest mutator *P. aeruginosa* isolates were found after 5 years from the onset of the chronic lung infection, and once they were present in the CF lung, the prevalence increased with time. The hypermutable isolates were significantly more resistant to antipseudomonal antibiotics than nonhypermutable isolates ( $P \leq 0.001$ ). The level of 8-oxodG/10<sup>6</sup> deoxyguanosine (dG) was significantly higher in hypermutable *P. aeruginosa* isolates ( $87 \pm 38$ ) than in nonhypermutable *P. aeruginosa* isolates ( $59.4 \pm 17$ ) ( $P = 0.02$ ), and an increase to 86.84 from 21.65 8-oxodG/10<sup>6</sup> dG was found after exposure of the reference strain PAO1 to activated PMNs. Our results suggest that the chronic PMN inflammation in the CF lung promotes oxidative stress and is associated with the occurrence of hypermutable bacteria in the lung. The hypermutable phenotype can associate with mutations that confer adaptation of the bacteria in the lung and persistence of the infection.**

The reduced volume of the epithelial lining fluid and viscous mucus in the lungs of patients with cystic fibrosis (CF) leads to a dysfunction of the mucociliary clearance, and as a consequence, the patients suffer from recurrent and chronic respiratory tract infections caused mainly by bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia*, and especially *Pseudomonas aeruginosa*.

A high percentage of *H. influenzae*, *S. aureus*, and *P. aeruginosa* strains isolated from patients with CF and chronic lung infection show higher frequencies of mutations ("hypermutable strains") than bacterial strains isolated from non-CF patients (26, 28a, 29a). Acquisition of hypermutability confers an advantage to the bacteria in stressful and fluctuating environments, as high mutation rates and recombination allow faster adaptation (17). The CF lung is a highly stressful and fluctuating environment, as, in addition to the challenge imposed by the host defenses, *P. aeruginosa* has to cope with the high doses of different antibiotics administered for prolonged periods of

time (25). The frequency of resistant strains was found to be roughly double in hypermutable strains than in nonhypermutable bacteria (26).

The chronic *P. aeruginosa* lung infection causes a chronic inflammatory response in the lung, dominated by polymorphonuclear leukocytes (PMNs). Analysis of bronchoalveolar lavage fluid has shown that the number of PMNs recovered from the lungs of patients with CF is 1,000 times higher than that recovered from the lungs of controls (2).

PMNs release leukocyte proteases, myeloperoxidase, and reactive oxygen species (ROS), which are the main mechanisms of lung tissue damage in CF (6, 13). The ROS liberated by leukocytes, together with the endogenous oxygen species that occur during replication, may increase the oxidative stress of the bacterial DNA. Oxidation of the guanine 8-oxo-2'-deoxyguanosine (8-oxodG) is a frequently encountered lesion. 8-oxodG promotes the misincorporation of adenine in the replication round, producing G:C to T:A transversions, thus making unrepaired 8-oxodG a highly mutagenic lesion.

Several mechanisms are involved in the repair of this mutagenic lesion. The main pathway for the removal of 8-oxodG in DNA involves the formamidopyrimidine DNA glycosylase (Fpg or MutM), encoded by the *mutM* gene (36). If the 8-

\* Corresponding author. Mailing address: University of Copenhagen, Panum Institute, IMMI 24.1, Blegdamsvej 3, 2200 Copenhagen N, Denmark. Phone: 45 35 32 78 99. Fax: 45 35 32 76 93. E-mail: O.Ciofu@immi.ku.dk.

oxodG is not removed from the DNA by Fpg and mispairs with an adenine, a mismatch-correction glycosylase, encoded by *mutY*, removes the mispaired adenine from the DNA (1). Besides the oxidation of DNA, other bacterial components can be oxidized, like the nucleotide pool. The oxidation product of GTP, 8-oxodGTP, is removed by hydrolysis from the nucleotide pool by a protein encoded by *mutT* (18).

Mutations in genes involved in the repair of the DNA oxidative damage (GO system) increase the number of 8-oxodG molecules and the mutation frequencies of the isolates (20, 21, 35).

Besides the repair of the oxidative damage, bacteria possess a major mismatch correction pathway, a methyl-directed pathway specified by proteins encoded by *mutS*, *mutL*, and *uvrD* (the MMR system). Inactivation of the MMR and/or the GO system can give rise to the mutator phenotype (22).

Our study tries to address the role of chronic inflammation in the occurrence of the mutagenic 8-oxodG lesion in *P. aeruginosa* isolates from CF patients and the implication for the occurrence of hypermutable isolates. To determine if there is any association between the chronic inflammation and the occurrence of hypermutable isolates, we analyzed in a longitudinal study the occurrence and the prevalence of hypermutable isolates during chronic lung infection with *P. aeruginosa*, and we determined in a cross-sectional study the frequency of hypermutable *P. aeruginosa* strains directly in the sputum of CF patients.

As a marker of the degree of oxidative damage of the DNA, we measured the concentration of 8-oxodG in hypermutable and nonhypermutable clinical isolates of *P. aeruginosa* and in the reference *P. aeruginosa* strain PAO1 exposed in vitro to activated PMNs.

## MATERIALS AND METHODS

**CF patients and *P. aeruginosa* isolates.** Three hundred CF patients, both children and adults, are monitored at the CF Center of Copenhagen University Hospital. All CF patients have been seen in the outpatient clinic on a monthly basis since 1968 (7).

Since 1976 all CF patients chronically infected with *P. aeruginosa* have been admitted every third month to the center for a 2-week course of intravenous antipseudomonal antibiotics.

*P. aeruginosa* isolates have been collected from the sputum of CF patients since 1973 and stored in nutrient broth containing 5% glycerol at  $-80^{\circ}\text{C}$ . The selection criteria were phenotypic (mucoid and nonmucoid) and different patterns of resistance to antibiotics, as determined on the basis of direct sensitivity testing of isolates from plated sputum. Chronic *P. aeruginosa* infection is defined as the continuous growth of *P. aeruginosa* for  $\geq 6$  months or the presence of two or more precipitating antibodies against *P. aeruginosa* (7).

(i) **Cross-sectional study.** To study the occurrence of hypermutable *P. aeruginosa* directly in the sputum bacterial population, sputum samples were collected from 79 consecutive CF patients with chronic lung infection during the period from October 2002 to March 2003 and were investigated further, as described below. The mean age of the patients was  $31.5 \pm 7.3$  years, and the mean duration of the chronic lung infection was  $21 \pm 6.8$  years.

(ii) **Longitudinal study.** To study the occurrence and the dynamics of the hypermutable *P. aeruginosa* isolates during the course of the chronic lung infection, 141 *P. aeruginosa* isolates collected from 1973 to 1999 from 11 CF patients were investigated.

For practical purposes the duration of the chronic lung infection was expressed in five 5-year periods, from 0 to 5, 5 to 10, 10 to 15, 15 to 20, and 20 to 25 years from the onset of the chronic lung infection.

**Determination of antibiotic resistance.** The in vitro activities of piperacillin-tazobactam, ceftazidime, aztreonam, meropenem, ciprofloxacin, tobramycin, and colistin (Rosco, Copenhagen, Denmark) against all *P. aeruginosa* isolates from

the cross-sectional and the longitudinal studies were tested by the diffusion method.

The MICs of piperacillin-tazobactam, ceftazidime, aztreonam, meropenem, ciprofloxacin, tobramycin, and colistin were determined by the agar plate dilution method, as described previously (5).

MIC determinations were done on all 141 *P. aeruginosa* isolates collected from 1973 to 1999 from 11 CF patients included in the longitudinal study, 100 *P. aeruginosa* isolates collected from the 43 CF patients with hypermutable isolates, and 100 isolates collected from the 36 CF patients without hypermutable isolates included in the cross-sectional study.

**Precipitating antibodies against *P. aeruginosa* in CF sera.** *P. aeruginosa* precipitins were determined by crossed immunoelectrophoresis, as described previously (11), with the normal range being zero to one precipitin.

**Measurement of mutation frequencies of *P. aeruginosa* isolates.** To determine the mutation frequencies after exposure to rifampin and streptomycin, the bacterial isolates were grown overnight in 20 ml Luria-Bertani (LB) medium, centrifuged at 3,000 rpm for 10 min, and resuspended in 1 ml LB medium. A 100- $\mu\text{l}$  volume of  $10^{-1}$ , and  $10^{-2}$  dilutions were plated on LB plates containing 300  $\mu\text{g/ml}$  rifampin and on LB plates containing 500  $\mu\text{g/ml}$  streptomycin. A 100- $\mu\text{l}$  volume of  $10^{-7}$  to  $10^{-10}$  dilutions were plated on LB plates, and the numbers of CFU were counted after incubation at  $37^{\circ}\text{C}$  for 48 h. An isolate was considered hypermutable if the mutation frequency after exposure to rifampin and streptomycin was 20 times higher than the mutation frequency of the reference strain PAO1, as published previously (26).

**Direct bacteriological examination of sputum.** Quantification of the *P. aeruginosa* isolates in the sputum samples was performed by a previously described population method (8). This technique allowed us to investigate the sputum samples directly without subculture and detect the presence of rifampin- and streptomycin-resistant isolates in the bacterial population from the sputum. In short, sputum samples collected from CF patients were stored at  $4^{\circ}\text{C}$ . All sputum samples were Gram stained and examined by microscopy in order to ensure that the samples were of sputum rather than saliva. Homogenization with Sputulycin (dithiothreitol; Sigma, St. Louis, Mo.) at a final concentration of 50 mg/liter was performed by a previously described method (10).

Sputum was diluted  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  in saline. An aliquot of 0.1 ml of the undiluted sputum and of the first two dilutions was inoculated on modified Conradi-Drigalski medium ("blue plates"; State Serum Institute, Copenhagen, Denmark) containing 300  $\mu\text{g/ml}$  rifampin and on plates containing 500  $\mu\text{g/ml}$  streptomycin, while 0.1 ml of the last three higher dilutions were inoculated on "blue plates" without antibiotics. After 48 h of incubation at  $35^{\circ}\text{C}$ , the *P. aeruginosa* colonies on the various plates were counted. For this study only CF sputum samples with  $\geq 10^4$  CFU/ml were considered. The frequency of rifampin- or streptomycin-resistant bacteria was calculated by dividing the number of colonies grown on plates containing antibiotics by the number of colonies grown on plates without antibiotics. Only plates containing  $\geq 20$  colonies/plate were considered.

The colonial morphotypes and the antibiotic resistance patterns were used to discriminate between several isolates. For each sputum sample at least two and up to six different isolates from plates with and without antibiotics were stored in nutrient broth containing 5% glycerol at  $-80^{\circ}\text{C}$ . For each patient, at least two isolates from plates with and without antibiotics, respectively, were tested for their mutation frequencies after exposure to rifampin and streptomycin, as described above.

**Typing of the isolates by PFGE.** The *P. aeruginosa* isolates included in the longitudinal study and the *P. aeruginosa* isolates collected from the cross-sectional study were typed by pulsed-field gel electrophoresis (PFGE), as described previously (24, 30), by using the Spe enzyme. The patterns were analyzed visually.

**Measurement of 8-oxodG in *P. aeruginosa* DNA.** (i) **DNA purification.** Bacterial DNA was purified with a PUREGENE DNA purification system (Gentra Systems, Minneapolis, Minn.), according to the instructions of the manufacturer, with some modifications.

Briefly, the bacterial lysate from an overnight culture (500  $\mu\text{l}$ ) was treated with RNase A solution (4 mg/ml) for 60 min at  $52^{\circ}\text{C}$ . After protein precipitation, the DNA was precipitated with 100% isopropanol and treated for a second time with RNase (10 mg/ml) for 60 min at  $52^{\circ}\text{C}$ . Sodium acetate (3 M; 10  $\mu\text{l}$ ; pH 5.2) was added, and the DNA was precipitated with 2 volumes absolute ethanol and washed with 70% ethanol. The purified DNA was resuspended in 10 mM Tris-0.1 mM desferrioxamine buffer, pH 7.

(ii) **DNA hydrolysis.** The purified DNA was hydrolyzed by nuclease P1 (1 U/ $\mu\text{l}$  in 30 mM sodium acetate, 1 mM  $\text{ZnCl}_2$ , pH 5.3; Z-0152; Sigma) for 120 min at  $37^{\circ}\text{C}$  and then incubated with alkaline phosphatase from *Escherichia coli* (1 U/ $\mu\text{l}$  in purified water; P-5931; Sigma) for 60 min at  $37^{\circ}\text{C}$ . The protein was extracted

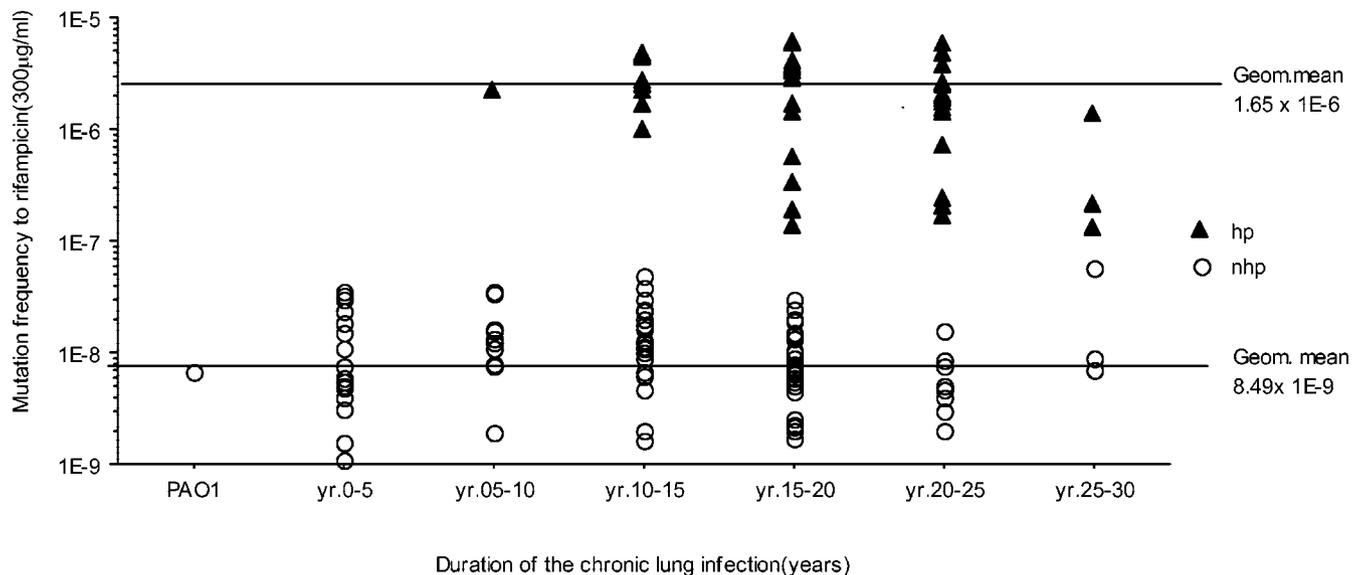


FIG. 1. Mutation frequency after exposure to rifampin of 141 *P. aeruginosa* isolates collected in five periods of chronic lung infection (0 to 5, 5 to 10, 10 to 15, 15 to 20, and 20 to 25 years) from 11 patients with CF. The results for hypermutable (hp; ▲) and nonhypermutable (nhp; ○) *P. aeruginosa* isolates are presented. The mutation frequency after exposure to rifampin of reference strain PAO1 is shown.

with 50  $\mu$ l chloroform, and the digested DNA was transferred to the analysis vials.

(iii) **Measurement of 8-oxodG.** Quantification of 8-oxodG was done by high-performance liquid chromatography with electrochemical and UV detection by use of a Prodigy 5  $\mu$ m octyldecyl silane column (Phenomenex, Torrance, Calif.) and 3% acetonitrile in phosphate buffer (pH 6) as the mobile phase. The samples were extracted in duplicate, and each was injected twice. The 8-oxodG was quantified in an electrochemical detector (Coulchem II and model 5011 Analytical Cell; both from ESA, Inc., Chelmsford, Mass.), while dG was quantified by determination of the UV absorbance (LaChrom UV detector L-7400; Merck Hitachi). Peak areas were used for the calculations. Separate calibration curves for 8-oxodG and dG were run together with each batch of samples.

The reproducibility of the assay for the measurement 8-oxodG was assessed by measuring in duplicate 10 different DNA purifications of reference strain PAO1. The variation coefficient was determined by using the formula standard deviation (SD) =  $\sqrt{\sum d^2/2n}$  (where  $\sum d^2$  is the sum of squared differences of double determinations of the same sample and  $n$  is the number of observations) and was found to be 2.1%.

**PMN bactericidal assay.** A bactericidal assay was performed by a previously described method (9). Human PMNs and the reference strain PAO1 at a ratio of 1:20 were incubated in the presence of 10% serum for 2 h at 37°C. The bacteria recovered from this experiment represented bacteria that survived phagocytosis (i.e., bacteria that were present both inside and outside the PMNs). In order to obtain only bacteria that survived inside the PMNs, the extracellular bacteria were killed by the addition of ceftazidime and tobramycin (200  $\mu$ g/ml) to another mixture of PMNs and bacteria after 15 min, and the tubes were further incubated for 2 h 45 min. The bacteria that survived the phagocytosis by PMNs were cultured overnight, and the DNA was purified as described above for the 8-oxodG measurements.

**Statistical analysis.** The description and analysis of the data were carried out by using StatView 5 software for personal computers. The data are given as means  $\pm$  SDs or geometric means. The unpaired *t* test was used for comparison of the data for the hypermutable isolates and the nonhypermutable isolates.

## RESULTS

**Hypermutable *P. aeruginosa* in the bacterial population from CF sputum samples (cross-sectional study).** The number of *P. aeruginosa* CFU in the 79 sputum samples was  $(1.4 \pm 5.6) \times 10^8$  CFU/ml sputum (mean value  $\pm$  SD).

The frequency of rifampin-resistant isolates in the sputum

samples was  $1.8 \times 10^{-5}$ , and that of streptomycin-resistant isolates was  $2 \times 10^{-5}$  (geometric mean).

The hypermutable phenotype of colonies isolated from the rifampin-containing plates was confirmed by testing the mutation frequencies after exposure to streptomycin.

Hypermutable *P. aeruginosa* isolates were found in the sputum of 43 of the 79 CF patients (54.4%). Genotyping showed that the rifampin- and streptomycin-resistant isolates and the sensitive subpopulation present in the sputum samples had similar PFGE patterns.

At the time of bacterial isolation from sputum, the 43 CF patients with hypermutable *P. aeruginosa* isolates were significantly older (mean age,  $33.7 \pm 6.5$  years), had significantly longer durations of chronic lung infection (mean duration,  $23.5 \pm 4.8$  years), and higher number of precipitins in serum (mean number,  $30 \pm 9$ ) compared to the 36 CF patients from whom hypermutable *P. aeruginosa* could not be isolated (mean age,  $29.3 \pm 7.5$  years; duration of chronic lung infection,  $18.4 \pm 7.7$  years; mean number of serum precipitins,  $26 \pm 11$ ) ( $P = 0.007$ ,  $P = 0.0007$ , and  $P = 0.036$ , respectively).

The MICs of *P. aeruginosa* isolates from the sputum of the 36 CF patients without hypermutable isolates to colistin (MIC<sub>50</sub> = 3.1  $\mu$ g/ml), piperacillin-tazobactam (MIC<sub>50</sub> = 25  $\mu$ g/ml), aztreonam (MIC<sub>50</sub> = 25  $\mu$ g/ml), and ceftazidime (MIC<sub>50</sub> = 12.5  $\mu$ g/ml) were significantly lower than the MICs of isolates from the sputum of the 43 CF patients with hypermutable isolates (MIC<sub>50</sub> of colistin = 6.2  $\mu$ g/ml, MIC<sub>50</sub> of piperacillin-tazobactam = 100  $\mu$ g/ml, MIC<sub>50</sub> of aztreonam = 100  $\mu$ g/ml, MIC<sub>50</sub> of ceftazidime = 200  $\mu$ g/ml).

**Hypermutable *P. aeruginosa* isolates during chronic lung infection (longitudinal study).** Hypermutable *P. aeruginosa* isolates were found in 8 of the 11 CF patients. Forty-three mutators were identified among the 141 *P. aeruginosa* isolates. The mutation frequency after exposure to rifampin was  $1.5 \times 10^{-6}$  (geometric mean), and that after exposure to streptomycin-

TABLE 2. Levels of 8-oxodG/10<sup>6</sup> dG in hypermutable and nonhypermutable *P. aeruginosa* isolates from CF patients<sup>a</sup>

CF patient no.	8-oxodG/10 <sup>6</sup> dG	
	Hypermutable isolates	Nonhypermutable isolates
CF 1	52	39.6
CF 2	62.3	60.1
CF 3	71.9	57.8
CF 4	70.2	40.8
CF 5	105.4	37.7
CF 5		49
CF 6	51.4	ND <sup>b</sup>
CF 6	70.6	
CF 6	90.2	
CF 7	50.3	ND
CF 7	56	
CF 8	117.9 <sup>c</sup>	75 <sup>d</sup>
CF 9	67.4 <sup>d</sup>	58.8 <sup>e</sup>

<sup>a</sup> For patients CF 1 to CF 7 the 8-oxodG levels were measured by the use of single isolates. These isolates were collected from the population analysis of the sputum samples (cross-sectional study). For patients CF 8 and CF 9 the levels are means of the levels for several isolates from the longitudinal study.

<sup>b</sup> ND, not determined.

<sup>c</sup> Mean for five isolates.

<sup>d</sup> Mean for four isolates.

<sup>e</sup> Mean for two isolates.

cin was  $1.4 \times 10^{-6}$  (geometric mean); these frequencies are similar to a previously published frequency of  $3.6 \times 10^{-6}$  (26).

In comparison, the mutation frequencies of the nonhypermutable isolates after exposure to rifampin and streptomycin were  $8.7 \times 10^{-9}$  and  $3.2 \times 10^{-8}$ , respectively.

The distribution of the hypermutable isolates in the five time periods of the chronic lung infection were 0%, 7%, 29%, 32%, and 65% (Fig. 1). The mean numbers of precipitating antibodies to *P. aeruginosa* in the patient's sera during the five time periods were 15, 28, 28, 33, and 38, respectively.

The cumulative numbers of 2-week antibiotic courses/patient received by the 11 CF patients during the treatment of the chronic lung infection in the five time periods were 17.5, 35.5, 58, 82, and 97, respectively.

Hypermutable isolates had significantly higher MICs than the nonhypermutable isolates against all the antibiotics tested except colistin ( $P \leq 0.001$ ) (Table 1).

Genotyping of the consecutive *P. aeruginosa* isolates showed that the CF patients maintained the same strain in their lungs during the chronic lung infection (data not shown).

**Bacterial DNA oxidation (measurement of 8-oxodG) in clinical *P. aeruginosa* isolates.** The levels of 8-oxodG/10<sup>6</sup> dG were measured in *P. aeruginosa* isolates with the hypermutable and the nonhypermutable phenotypes but with identical genotypes. The measurements were done with isolates from the sputum samples of seven CF patients (from rifampin- and streptomycin-containing plates and from control plates) and 15 isolates collected during the chronic lung infection from two CF patients (Table 2).

Large variations in the levels of 8-oxodG were found among different *P. aeruginosa* isolates, but the hypermutable isolates showed significantly higher ( $P = 0.02$ ) levels of 8-oxodG/10<sup>6</sup> dG ( $87 \pm 38$ ) than the nonhypermutable isolates ( $59.4 \pm 17$ ), which was sign of the oxidative damage of their DNA.

TABLE 1. MIC<sub>50s</sub>, MIC<sub>90s</sub>, and MIC ranges for various antimicrobial agents of *P. aeruginosa* isolates with hypermutable and nonhypermutable phenotypes<sup>a</sup>

Phenotype	Ceftazidime <sup>b</sup>		Piperacillin-tazobactam <sup>b</sup>		Aztreonam <sup>b</sup>		Meropenem <sup>b</sup>		Tobramycin <sup>b</sup>		Colistin		Ciprofloxacin <sup>b</sup>	
	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%
Hypermutable	200	400	1.6-400	400	400	400	400	400	0.4-400	12.5	25	1.6-50	0.8	5
Nonhypermutable	6.2	200	0.8-400	100	400	400	0.8-400	12.5	50	0.1-200	3.1	12.5	0.4-25	1.6
			Range	Range	Range	Range	Range	Range	Range	Range	Range	Range	Range	Range

<sup>a</sup> Hypermutable isolates had frequencies of mutations after exposure to rifampin and streptomycin of  $1.6 \times 10^{-6}$  and  $1.4 \times 10^{-6}$ , respectively. For the nonhypermutable isolates, the frequencies were  $9 \times 10^{-9}$  and  $3.3 \times 10^{-8}$ , respectively.

<sup>b</sup> Statistically significant difference ( $P < 0.001$ ) between the MICs for the hypermutable and nonhypermutable isolates.

**Mutation frequency of *P. aeruginosa* reference strain PAO1 exposed to activated PMNs.** Exposure of PAO1 to activated PMNs led to a slight increase in the mutation frequency after exposure to rifampin, from  $3 \times 10^{-9}$  for the controls to  $7.4 \times 10^{-9}$  for the bacteria that survived intra- and extracellularly and to  $1.2 \times 10^{-8}$  for the bacteria that survived intracellularly.

**Bacterial DNA oxidation (measurement of 8-oxodG) in *P. aeruginosa* reference strain PAO1 exposed to activated PMNs.** Exposure of reference strain PAO1 to PMNs in a bactericidal assay led to a 90% reduction in the bacterial count. Higher levels of 8-oxodG/ $10^6$  dG were measured for the DNA of PAO1 isolates that survived the phagocytosis outside and inside the PMNs (37.78) and for the DNA of PAO1 isolates that survived in the PMNs (40.39) than for the DNA of the control (21.65).

Reexposure to PMNs of the PAO1 isolates that have survived inside the PMNs increased the levels of 8-oxodG/ $10^6$  dG in the bacteria present outside and inside the PMNs to 61.95 and in bacteria recovered from the PMNs to 86.84, which shows the increased oxidative damage to the bacterial DNA.

## DISCUSSION

We demonstrated an association between oxidative DNA modifications in *P. aeruginosa* and the development of hypermutable bacteria resistant to antibiotics. This confirms that the chronic lung infection in CF patients is a state of chronic oxidative stress (16, 37) that promotes DNA changes in the *P. aeruginosa* genome, which is linked to an increased possibility of adaptation in the CF lung, for example, the development of resistance to antibiotics, and, in addition, to growth in biofilms and to the production of alginate (19).

Investigation of the occurrence of mutators directly in the *P. aeruginosa* population present in the CF sputum showed that more than half of the 79 Danish CF patients included in the study had hypermutable isolates in their lungs. This proportion is higher than the proportion of 36% of 30 CF patients described by Oliver et al. (26). This difference can be explained by the different methods for the detection of hypermutable isolates used, namely, population analysis in our study and the detection of mutation frequencies among the isolated colonies in the study of Oliver et al. (26). The long duration of the chronic lung infection in the CF population included in our study might also explain the higher proportion of patients with hypermutable isolates compared to the proportion published previously (26).

The earliest hypermutable *P. aeruginosa* isolates occurred after a relatively long period of time from the onset of the chronic lung infection (5 years). It has been shown that the rate of PMN oxidative burst in response to a biofilm is reduced to about 30 to 80% of the response to planktonic bacteria (14). Moreover, it has also been shown that  $H_2O_2$  cannot penetrate to the bottom of a *P. aeruginosa* biofilm (34). Thus, the biofilm-growing bacteria in the CF lung might need to be exposed to oxygen radicals from the PMNs for a relatively long period of time before an effect on hypermutability can be measured. The detection of hypermutable isolates after 5 years of chronic lung infection might be also a consequence of the criteria used for the selection of the isolates included in the longitudinal study, namely, the mucoid or nonmucoid phenotype and their resis-

tance to antibiotics. In the beginning of the chronic phase of infection there were no or few resistant strains, but due to the development of antibiotic resistance, the number of resistant strains increased with time (4). However, once they were present in the CF lung, the prevalence of hypermutable *P. aeruginosa* isolates increased during the chronic lung infection. Our results are in agreement with the anticipated accumulation of mutants that occur in the course of chronic infection due to bacterial replication (27).

It is important that stable hypermutability does not confer an advantage by itself, due to the high number of deleterious mutations, but the association (hitchhiking) with rare favorable mutations confers a survival advantage (29).

Both in the cross-sectional and in the longitudinal studies, hypermutable isolates had significantly higher MICs of antipseudomonal antibiotics compared to those of nonhypermutable isolates. These data support previously published results reporting that resistance to antibiotics was roughly twofold greater in frequency in hypermutable strains than in nonhypermutable strains (26).

The number of serum precipitating antibodies to *P. aeruginosa*, which correlates to inflammatory parameters (12), also increased during the chronic infection, which suggests that the development of hypermutable isolates is associated with the degree of inflammation in the CF lung. This is also supported by the results of the cross-sectional study, in which we found that CF patients with *P. aeruginosa* hypermutable isolates in their sputum were significantly older, had a longer duration of chronic lung infection, and a higher number of precipitating antibodies to *P. aeruginosa* compared to the age, duration of infection, and number of precipitating antibodies in the group of patients from whom no hypermutable isolates were found.

The level of oxidative damage of the bacterial DNA was increased in the hypermutable *P. aeruginosa* isolates compared to that in the nonhypermutable *P. aeruginosa* isolates, as shown by measurement of the levels of 8-oxodG/ $10^6$  dG. In addition, in vitro exposure of reference strain PAO1 to activated PMNs led to increased levels of DNA oxidation after exposure and reexposure to PMNs compared to those for the controls.

In PAO1 the repair mechanisms of the DNA oxidative lesions are functional, and the high levels of 8-oxodG measured are probably due to the overburden imposed by the ROS liberated by PMNs to the repair system. After a single exposure of 2.5 h to PMNs, a slight increase in the mutation frequencies of strain PAO1 was observed, probably due to unrepaired 8-oxodG lesions.

Measurements of DNA oxidation in the hypermutable and nonhypermutable isolates showed no linear correlation between the levels of 8-oxodG and the mutation frequency. Hypermutability requires the occurrence of mutations in the genes that have been shown to be responsible for DNA repair, the GO system (*mutT*, *mutY*, and *mutM*) and/or the MMR system (*mutS*, *mutL*, and *uvrD*) (22). It has been shown in *E. coli* that inactivation of *mutM*, *mutY*, and *mutT* gives rise to weak, moderate, and strong mutator phenotypes, respectively, while inactivation of the MMR system genes gives rise to the strong mutator phenotype.

Oliver et al. (25) showed that alterations in the genes of the MMR system were responsible for the mutator phenotype in 7 of 11 mutator *P. aeruginosa* strains from CF patients. Comple-

mentation of the same 11 mutator *P. aeruginosa* strains with cloned *mutT* and *mutM*, not surprisingly, failed to decrease the mutation frequencies (28). However, mutator *P. aeruginosa* CF isolates in which *mutY* genes failed to be amplified by PCR were also reported by the same group (26). The coexistence of alterations in both MMR and GO systems in strong mutators cannot be excluded. A mutator cascade, in which one type of mutator (*mutT*) generates a second mutator (*mutHLS*), which then allows stepwise frameshift mutations, has been described in *E. coli* (23).

We hypothesize that oxidation of DNA by activated PMNs during chronic inflammation in the CF lung is an initial event in the development of a hypermutable strain.

Strains with high levels of 8-oxodG will have an increased risk of occurrence of mutations in genes involved in DNA repair and, subsequently, the emergence of hypermutable isolates.

Different hypermutable isolates from the same patient presented various levels of 8-oxodG. This probably reflects the heterogeneity of the *P. aeruginosa* strains in the CF lung, in which bacteria adapted to the different macrocompartments in the lung and the microcosmos of the biofilm are present.

The association of hypermutability with mutations in genes that improve the adaptation of the bacteria and the presence of selective pressure are necessary for the survival of these strains.

These data suggest that the chronic PMN inflammation in the lungs of CF patients may contribute to the occurrence of hypermutable isolates in this group of patients.

Our hypothesis may have implications for other conditions characterized by chronic lung inflammation, such as chronic obstructive pulmonary disease (COPD). In COPD the ROS from inflammatory cells as well as from the inhaled cigarette smoke play a major role in the pathogenesis and progression of this disease (32). Patients with COPD have chronic lung colonization with *H. influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*; and other pathogens, like *S. aureus*, *P. aeruginosa*, and members of the family *Enterobacteriaceae*, can also be isolated.

Chronic colonization of the lower respiratory tract by bacterial pathogens amplifies the chronic inflammatory response present in COPD and leads to progressive airway obstruction (32). The persistence of the same bacterial strains in the lungs of patients with COPD is a consequence of the good adaptation of the bacteria in the lungs, and we suggest that this might be due to the occurrence of hypermutable strains under the oxidative stress present in the lungs of patients with COPD.

An obvious strategy for prevention of the occurrence of hypermutable isolates due to the oxidative damage is the use of antioxidants. Chopra et al. (3) showed that addition of antioxidants to cultures of hypermutable *E. coli* reduced the mutation frequency.

The oxidative stress in the CF lung is determined by the large number of activated PMNs in the respiratory airways and also by the deficiency in the antioxidant systems, like reduced amounts of glutathione. It has been shown that the administration of glutathione aerosols to patients with CF suppresses the oxidative burden on the surface of lung epithelial cells (31). We have previously shown that *N*-acetylcysteine can decrease the oxidative burst of PMNs and monocytes and that this drug

has a positive influence on the clinical condition of CF patients with chronic *P. aeruginosa* infection (15, 33)

Treatment with antioxidants would probably also decrease the oxidative damage to the bacterial DNA and prevent the occurrence of hypermutable isolates.

Our results support the hypothesis that the chronic PMN inflammation in the lungs of patients with CF may contribute to the occurrence of *P. aeruginosa* hypermutable isolates that play an important role in the development of resistance to antibiotics under the selection pressure of the heavy use of antibiotics in these patients. The use of early and aggressive antibiotic treatment for prevention of the chronic infection with *P. aeruginosa* and therapeutic approaches with antioxidants such as *N*-acetylcysteine to prevent the development of hypermutable bacteria during the chronic lung infection should be considered in the treatment of patients with CF.

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