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Antibiotic Resistance in *Pseudomonas aeruginosa* Strains with Increased Mutation Frequency Due to Inactivation of the DNA Oxidative Repair System[▽]

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The chronic *Pseudomonas aeruginosa* infection of the lungs of cystic fibrosis (CF) patients is characterized by the biofilm mode of growth and chronic inflammation dominated by polymorphonuclear leukocytes (PMNs). A high percentage of *P. aeruginosa* strains show high frequencies of mutations (hypermutators [HP]). *P. aeruginosa* is exposed to oxygen radicals, both those generated by its own metabolism and especially those released by a large number of PMNs in response to the chronic CF lung infection. Our work therefore focused on the role of the DNA oxidative repair system in the development of HP and antibiotic resistance. We have constructed and characterized *mutT*, *mutY*, and *mutM* mutants in *P. aeruginosa* strain PAO1. The *mutT* and *mutY* mutants showed 28- and 7.5-fold increases in mutation frequencies, respectively, over that for PAO1. These mutators had more oxidative DNA damage (higher levels of 7,8-dihydro-8-oxodeoxyguanosine) than PAO1 after exposure to PMNs, and they developed resistance to antibiotics more frequently. The mechanisms of resistance were increased β-lactamase production and overexpression of the MexCD-OprJ efflux-pump. Mutations in either the *mutT* or the *mutY* gene were found in resistant HP clinical isolates from patients with CF, and complementation with wild-type genes reverted the phenotype. In conclusion, oxidative stress might be involved in the development of resistance to antibiotics. We therefore suggest the possible use of antioxidants for CF patients to prevent the development of antibiotic resistance.

The chronic *Pseudomonas aeruginosa* lung infection in patients with cystic fibrosis (CF) is characterized by the biofilm mode of growth, which protects the bacteria against antibiotics and the innate and adoptive defense mechanisms (1, 14, 22). Intensive antibiotic treatment has improved the survival and clinical condition of CF patients, but the development of resistance to antibiotics makes these infections difficult to treat efficiently (20). Chronic inflammation in the lungs is characteristic for CF patients, and the immune responses are dominated by neutrophil polymorphonuclear leukocytes (PMNs), releasing proteases and oxygen radicals, which are the main cause of tissue damage in the lungs of CF patients (7). It has been shown that activated PMNs can cause oxidative stress and damage in patients with CF (6, 25, 59). The degrees of protein oxidation and lipid peroxidation in bronchoalveolar lavage fluid have been found to be significantly higher for CF patients than for healthy subjects (5, 57). Reactive oxygen species (ROS) have been shown to have a mutagenic effect on the bacterial DNA, leading to alginate production and biofilm formation (38), as well as to a higher mutation frequency (MF) and increased antibiotic resistance (9). It is assumed that bacteria with defects in their DNA repair pathways have a reduced ability to repair DNA damage and are more likely to accumulate mutations. Mutators are risk factors during the treatment of bacterial infections, because they appear to enhance the selection of mutants expressing high- and low-level antibiotic resistance (8).

The mutator phenotype is often seen to evolve through mutations in the genes responsible for DNA repair (13, 41, 43, 47, 49). It has also been reported that mutator strains are more frequently multidrug resistant than nonmutators (4, 34, 44). In recent years, much attention has been paid to hypermutability, since *P. aeruginosa* isolates from patients chronically infected with CF and other chronic obstructive lung diseases are more often found to have a mutator phenotype than isolates from other sources (9, 34, 45, 48).

Hypermutator (HP) strains were also significantly more resistant to antipseudomonal antibiotics than nonhypermutable isolates. Further, it was also found that HP isolates had higher levels of 7,8-dihydro-8-oxodeoxyguanosine (8-oxodG) than nonhypermutable isolates (9). The increased mutation rate helps HP isolates to fit better into new niches, such as the stressful CF lung environment (60). It seems that continued antibiotic treatment can select for hypermutability due to "hitchhiking" with mutations conferring antibiotic resistance (4). It has been shown that the majority of HP isolates have mutations in the DNA methyl-directed mismatch repair (MMR) system, particularly in the *mutS* gene (30, 31, 39, 47). Little is known about the role played by the DNA oxidative repair (GO) system in the occurrence of the hypermutable phenotype in *P. aeruginosa*. However, the role of the oxidative repair enzymes has been investigated with different bacteria, and these studies have shown that a lack of the enzymes in-

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volved in the GO system resulted in elevated MFs (13, 41). The system has been well characterized in *Escherichia coli* and recently also in *Pseudomonas putida*, and *mutY* has been characterized in *Helicobacter pylori* (13, 24, 53, 54). The enzymes of the GO repair system repair an oxidatively damaged form of guanosine (8-oxodG) and prevent its incorporation into DNA. This oxidative lesion is mutagenic due to its ability to base pair with either adenine or cytosine incorporated into DNA or found in the nucleotide pool. The GO system in *E. coli* consists of several genes encoding the repair enzymes: MutT is a hydrolase that converts 8-oxodGTP to 8-oxodGMP and PP_i, preventing oxidized guanine from being incorporated during replication and giving rise to $A \cdot T \rightarrow C \cdot G$ transversions. MutM is an N glycosylase that removes 8-oxodG when it is base paired with cytosine, and MutY is an adenine glycosylase that removes adenine base paired with 8-oxodG, both leading to increases in $G \cdot C \rightarrow T \cdot A$ transversions. GO mutants are expected to specifically increase the rate of $G \cdot C \rightarrow T \cdot A$ or $A \cdot T \rightarrow C \cdot G$ transversions (12, 13, 42).

Oliver et al. have found that *P. aeruginosa* has an oxidative repair system homologous to the GO system described for *E. coli*. The *mutY*, *mutT*, and *mutM* genes of PAO1 were characterized by cloning, sequencing, and complementation of the mutations in the corresponding deficient *E. coli* strains (49).

To investigate the impact of the *P. aeruginosa* GO system on the mutator phenotype, we constructed PAO1 *mutY*, *mutM*, and *mutT* mutants. Several studies have focused on the role of the MMR genes in the development of HPs and the development of resistance. The aim of this study was to investigate the development of resistance to antibiotics in clinical CF *P. aeruginosa* isolates and in PAO1 GO-deficient mutants with impaired oxidative repair mechanisms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All strains and plasmids included in the present study are described in Table 1. As a reference strain we used PAO1. The bacteria were grown in Luria-Bertani (LB) broth or LB agar containing the appropriate antibiotics.

Construction of insertion inactivation mutants. *P. aeruginosa* PAO1 *mutT*, *mutY*, and *mutM* mutants were generated by using a gene replacement strategy (55). The three genes were amplified from a PAO1 template by PCR with primers mutT-out-FW1 and mutT-out-rv1, mutY-out-fw1 and mutY-out-rv1, and mutM-out-rv1 and mutM-out-fw1, respectively (Table 1). The three genes were separately cloned into the SmaI restriction site of pUC18not transformed into competent *E. coli* JM105 and selected on AXI plates (LB agar with 0.1 mM isopropyl-β-D-thiogalactopyranoside [IPTG], 0.1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal], and 100 μg/ml ampicillin). Insertion of a gentamicin cassette flanked by SmaI into each of the three genes in pUC18not makes them nonfunctional. pUC18not::*mutT* was digested by ClaI; pUC18not::*mutY* was digested by EcoNI; and pUC18not::*mutM* was digested by SacII. The ends were blunted with Klenow enzyme (Roche, Mannheim, Germany) and dephosphorylated before they were ligated with the gentamicin cassette, and the plasmids were once again transformed into competent *E. coli* JM105. The plasmids were digested with NotI flanking the whole insert of the Δ*mut* "gene" ligated to the gentamicin cassette. This fragment was ligated into the NotI site of the gene replacement vector pCK318s. The three respective vectors were transformed into competent *E. coli* cc118 λ-pir cells and selected on LB agar containing 15 μg/ml gentamicin. The plasmids were digested with NotI to ensure that the insert was correct. *E. coli* s17 λ-pir containing pCK318-Δ*mutT*::Gm, pCK318-Δ*mutY*::Gm, or pCK318-Δ*mutM*::Gm was used as the donor strain in diparental mating with *P. aeruginosa* PAO1. Transconjugants were selected on *Pseudomonas* isolation agar plates (6.7 g tryptone, 1.65 g yeast extract, 0.85 g $NaCl_2$, 10 g agar, 6.3 g *Pseudomonas* isolation agar [Difco, Sparks, MD], and 1 liter MilliQ water) containing 60 μg/ml gentamicin and were subsequently plated on LB agar containing 60 μg/ml gentamicin and 5% sucrose to select for successful double-crossover events leading to insertion of the gentamicin cassette disrupting the three respective *mut* genes.

PCR amplification confirmed that the gentamicin cassette was inserted into the three respective *mut* genes in PAO1. Of the primers used for checking the mutants, mutY-fw-tjek and mutY-rev-tjek yielded LM118, mutT-fw-tjek and mutT-rev-tjek yielded LM117, and mutM-fw-tjek and mutM-rev-tjek yielded mutant LM121 (Table 1).

Construction of complementation plasmids. The *P. aeruginosa–E. coli* shuttle vector pUCP26 (62) was used for the construction of recombinant plasmids containing the PAO1 *mutT*, *mutY*, and *mutM* genes under the control of the plasmid-borne *lac* promoter. These plasmids were constructed by amplifying *mutT*, *mutY*, and *mutM* from PAO1 templates with primers mutT fw m. BamHI+SD and mutT rev m. HindIII, mutY fw m. BamHI+SD and mutY rev m. HindIII, and mutM fw m. XbaI+SD and mutM rev m. HindIII, respectively (Table 1). The primers were provided with restriction sites matching those in the pUCP26 multicloning site and a Shine-Dalgarno motif, resulting in recombinant plasmids pLM100, pLM101, and pLM102 (40).

P. aeruginosa was transformed with pLM100, pLM101, and pLM102 by electroporation. The transformed bacteria were inoculated onto LB agar containing 80 μg/ml tetracycline or 80 μg/ml tetracycline plus 0.1 mM IPTG and were incubated for 24 h to select the transformants LM76, LM79, and LM82.

Growth rates. PAO1 and GO mutants were grown overnight in LB medium, and each culture was standardized to an optical density at 600 nm (OD_{600}) of 0.01. Bacterial growth at 37°C was measured every 20 min until stationary phase was reached. The OD_{600}s determined were plotted against time in a semilogarithmic graph, and the doubling time was determined from the slope of a straight line during exponential growth. The doubling time is the mean of results for three individual cultures ± the standard deviation.

MF measurement. The method for measuring the MF was modified from that of Oliver et al. (48) To determine the MFs in response to rifampin (rifampicin) and streptomycin, an overnight culture in 20 ml LB medium was centrifuged for 10 min at 6,000 × *g* and resuspended in 1 ml of 0.9% $NaCl_2$. Portions (100 μl) from this suspension and successive dilutions were plated onto LB plates as well as onto LB plates containing 300 μg/ml rifampin or 500 μg/ml streptomycin. The CFU was counted after incubation at 37°C for 48 h, and the ratio between colonies on LB agar and antibiotic plates refers to the MFs. These experiments were reproduced at least three times.

It has been proposed that the MF of a *P. aeruginosa* isolate has to be at least 20-fold higher than the MF of PAO1 in order for the isolate to be defined as a mutator (48). In this study we consider isolates for which the MF increased <5-fold over that for PAO1 to be weak mutators, those for which the MF increased 5- to 10-fold to be moderate mutators, and those for which the MF increased at least 20-fold to be strong mutators.

Mutation rate. The mutation rates were estimated by using a fluctuation experiment where a culture of each strain was diluted to 2×10^4 cells in 280 μl LB medium, grown in 27 microtiter wells to stationary phase, and then plated onto LB agar plates with 100 μg/ml rifampin in order to count the number of mutants. Three wells for each strain were used to estimate the CFU per well. The expected number of mutations per well was then estimated using the MSS maximum-likelihood method described by Ma et al. (33). The mutation rate was found by dividing the number of mutations by the final CFU per well. For PAO1, two independent experiments with different final CFU counts were used, and here the estimation was performed directly on the mutation rate.

Determination of antibiotic susceptibility. MICs were determined by using the Etest system (AB Biodisk, Solna, Sweden) according to the instructions of the manufacturer. The disk diffusion method was used. Overnight cultures of bacteria diluted 10^{-2} (10^8 cells/ml) were added before the antibiotics (Neo-Sensitabs; Rosco, Copenhagen, Denmark). The plates were incubated at 37°C for 20 h.

To establish the compositions of the bacterial populations of the three GO mutants, population analyses against four different antibiotics—piperacillin-tazobactam (Wyeth Lederle), ceftazidime (Glaxo Wellcome), tobramycin (Sygehus apotekerne i Danmark), and ciprofloxacin (Bayer)—were done. Population analyses were performed as described previously (2). The overnight cultures were diluted and plated onto 5% blood agar plates containing twofold dilutions of the respective antibiotics. The CFU on plates containing different antibiotic concentrations were counted and compared with the CFU from plates without antibiotics, and the percentages of survival of the different bacterial populations were calculated.

Real-time PCR. The levels of expression of *mexB*, *mexD*, *mexF*, and *mexX* were determined by real-time PCR. Resistant colonies from the ciprofloxacin and tobramycin antibiotic plates used for population analysis were picked, and the antibiotic susceptibility profiles of the selected mutants were studied after three passages in antibiotic-free LB medium.

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TABLE 1. Strains, plasmids, and primers

Strain, plasmid, or primer	Genotype, characteristics, or sequence[a]	Reference or source
Strains		
P. aeruginosa PAO1		58
E. coli JM105		Pharmacia
E. coli cc118 λ-pir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoE*(Am) *recA1*; lysogenized with Δ*pir* phage	18
E. coli s17 λ-pir	*thi pro hsdR recA* RP4-2 (Tet::Mu) (Km::Tn7) λ	56
LM118	PAO1 Δ*mutY*::Gm	This study
LM117	PAO1 Δ*mutT*::Gm	This study
LM121	PAO1 Δ*mutM*::Gm	This study
LM76	PAO1 Δ*mutY*::Gm with pLM100	This study
LM79	PAO1 Δ*mutT*::Gm with pLM101	This study
LM82	PAO1 Δ*mutM*::Gm with pLM102	This study
Plasmids		
pUC18not	Ap^r; identical to pUC18 but with NotI/polylinker of pUC18/NotI as MCS	18
pCK318	RP4*mob ori*R6K *sacB bla*	16
pUCP26	*P. aeruginosa–E. coli* shuttle vector	62
pLM100	pUCP26 containing 1.2-kb BamHI-HindIII PCR fragment of PAO1 *mutY*	This study
pLM101	pUCP26 containing 1-kb BamHI-HindIII PCR fragment of PAO1 *mutT*	This study
pLM102	pUCP26 containing 0.9-kb XbaI-HindIII PCR fragment of PAO1 *mutM*	This study
pLM103	pUCP26 containing 1.95-kb BamHI-HindIII PCR fragment of PAO1 *mutL*	This study
Primers for PCR		
mutY-fw-tjek	5′-GACAAGGAAGGCATGGGCAAGGTC-3′	This study
mutY-rev-tjek	5′-GGTACTTGCGGCACATCACGGTG-3′	This study
mutM-fw-tjek	5′-CGACTACGACTTCGAGAACCTCAAGG-3′	This study
mutM-rev-tjek	5′-GGACATCGTGCACCTTTCTTATGGCAG-3′	This study
mutT-fw-tjek	5′-TATGTCGAGACGATTTATCAGTGGCCTG-3′	This study
mutT-rev-tjek	5′-GACCAGGAGGAAACGCTGGAGG-3′	This study
nfxB fw 1	5′-GCACAATGCGCACAATCAG-3′	This study
nfxB rev 1	5′-TCGGTCCGTGCCATGC-3′	This study
Primers for complementation		
mutY fw m. BamHI+SD	5′-CGCGGATCCGCGAGGAGAAGAGCTA CGCAAATGACACCTGAAGGC-3′	This study
mutY rev m. HindIII	5′-CCCAAGCTTGGGTCATGGTCGTTCTCCTGC-3′	This study
mutM fw m. XbaI+SD	5′-GCTCTAGAGCAGGAGAAGTGCAATGCG CATGCCCGAACTACCCGAAG-3′	This study
mutM rev m. HindIII	5′-CCCAAGCTTGGGCTTCTTGTGGCGGTAGAGTATGC-3′	This study
mutT fw m. BamHI+SD	5′-CGCGGATCCGCGAGGAGAAGTGCAATGC CCGTGAAACGAGTACATGTC-3′	This study
mutT rev m. HindIII	5′-CCCAAGCTTGGG TTCATTCCACCGTCAAAGGC-3′	This study
mutL fw. m. BamHI+SD	5′-CGCGGATCCGCG AGGAGAAG TTCA CCAGTGATGAGTGAAGCACC-3′	This study
mutL rev m. HindIII	5′-CCCAAGCTTGGG GAAGCTGCAAGGCTCAGA-3′	This study
Primers for RT-PCR		
MexF-L RTpcr	5′-TCTACGACCCGACCATCTTC-3′	This study
MexF-R RTpcr	5′-CAGGTCTGCAGGAACAGGAT-3′	This study
MexY-L RTpcr	5′-GCCCAACGACATCTACTTCAA-3′	This study
MexY-R RTpcr	5′-CATGCCTTCCTGGTAATGGT-3′	This study
MexD-L RTpcr	5′-TCAACGGTCTGGGTAACTCC-3′	This study
MexD-R RTpcr	5′-TGGATCTCGCCAAGAAGAGT-3′	This study
MexB-L RTpcr	5′-TACGAAAGCTGGTCGATTCC-3′	This study
MexB-R RTpcr	5′-GAAGAACACGTCGTTGGACA-3′	This study

[a] MCS, multiple cloning site. Underlined nucleotides in sequences of primers for complementation are Shine-Dalgarno motifs.

RNA from the logarithmic-growth phase (OD_{600}, 0.9) was purified with the RNeasy minikit (Qiagen, Hilden, Germany), followed by a DNase treatment with RQ1 RNase-free DNase (Promega). Purified RNA (280 ng) was then used for one-step reverse transcription and real-time PCR amplification using a QuantiTect reverse transcription kit and a SYBR green PCR kit (Qiagen, Hilden, Germany) in the PCR Mx3005P real-time PCR system. Amplification was performed with the primers reported in Table 1 and the following protocol: 15 min at 95°C, followed by 40 cycles of 20 s at 95°C, 20 s at 60°C, and 30 s at 72°C. A melting curve was run at the end to test for the presence of a unique PCR product. The ribosomal *rpsL* gene was chosen as the reference gene.

β-Lactamase assay. Resistant colonies from the ceftazidime antibiotic plates used for the population analysis were picked up, and the antibiotic susceptibility profiles of the selected colonies were studied after three passages in antibiotic-free LB medium. The basal β-lactamase level and the level after 2.5 h of induction with benzylpenicillin (500 μg/ml) were measured spectrophotometrically with nitrocefin (51.6 μg/ml) as the substrate as previously described (46).

PMN assay. A bactericidal assay was performed by a method previously described (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 were those that avoided or survived phagocytosis (i.e., bacteria that were

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TABLE 2. Overview of the MFs, mutation rates, and levels of resistance of the GO mutants

Mutant	Doubling time (min)	Mutation rate	Mean MF in response to:		MIC (mg/liter) by Etest[a] (size of resistant mutant subpopulation[b])				
			Rifampin (300 μg/ml)	Streptomycin (500 μg/ml)	PIP	CAZ	TOB	CIP	ATM
PAO1	26 ± 0.6	6.75E-09	1.81E-8	8.48E-10	2 (+)	2	1.5	0.19	1.5
PAO1/pUCP26			1.37E-8	5.38E-10					
PAO1Δ*mutY*::Gm	25.7 ± 1.7	3.85E-08	1.36E-7	3.51E-9	2 (++)	1.5 (++)	1.5 (+)	0.19 (+)	1 (+)
PAO1Δ*mutY*::Gm/pLM100			6.36E-8	1.29E-9					
PAO1Δ*mutT*::Gm	27.7 ± 1.8	1.28E-07	5.07E-7	1.18E-7	2 (+++)	2 (+++)	1.5 (++)	0.25 (++)	1.5 (++)
PAO1Δ*mutT*::Gm/pLM101			3.91E-8	1.69E-9					
PAO1Δ*mutM*::Gm	27.3 ± 1	6.38E-09	2.78E-8	1.69E-9	2 (++)	1.5 (+)	1.5	0.19	1.5
PAO1Δ*mutM*::Gm/pLM102			2.53E-8	3.72E-10					

[a] PIP, piperacillin; CAZ, ceftazidime; TOB, tobramycin; CIP, ciprofloxacin; ATM, aztreonam.
[b] The numbers of resistant mutant colonies in the inhibition zone are as follows: +, <10; ++, 10 to 100; +++, >100 (35).

present either inside or 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 μg/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 phagocytosis by PMNs were cultured overnight, and the DNA was purified as described below.

Sequencing of the oxidative repair genes of clinical isolates. We have previously published the 8-oxodG levels of 31 clinical *P. aeruginosa* isolates from nine CF patients (9). We chose to sequence the oxidative repair genes in 16 isolates with high 8-oxodG levels. A high 8-oxodG level was defined as the 8-oxodG of PAO1 plus 2 standard deviations. Eleven of these isolates had a hypermutable phenotype, and five had a nonhypermutable phenotype. DNA was purified with a Promega (Madison, WI) Wizard purification kit. PCR amplification was performed with the PCR primers described in Table 1, and DyNAzyme EXT DNA polymerase (Finnzymes, Espoo, Finland) was used. Sequencing was done on an ABI 3700 automatic DNA sequencer (Macrogen Inc., Seoul, South Korea). The number of reads was between two and four for each gene of each strain. The sequence results were compared with the sequence of strain PAO1 (www.pseudomonas.com) with DNASIS MAX, version 2.0 (Hitachi Software Engineering), in order to determine the occurrence of sequence variants within the *mutT*, *mutY*, or *mutM* gene.

Sequencing of *nfxB* in strains hyperexpressing *mexD*. PCR amplification (Table 1) and sequencing were carried out as described above.

Measurement of 8-oxodG levels in *P. aeruginosa* DNA. The levels of 8-oxodG in bacterial DNA were estimated as previously described (9). DNA was purified using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions until point 4 in DNA precipitation, when the DNA was RNase treated an extra time with 10 μg/ml RNase A for 60 min at 52°C. Ten microliters of 3 M sodium acetate (pH 5.2) was added, and the DNA was precipitated with 2 volumes of absolute ethanol and washed with 70% ethanol. The purified DNA was resuspended in 10 mM Tris–0.1 mM desferoxamine buffer, pH 7, and stored at −80°C.

The purified DNA was hydrolyzed by nuclease P1 (Sigma Z-0152) (1 U/μl in 30 mM sodium acetate–1 mM $ZnCl_2$ [pH 5.3]) for 120 min at 37°C. The protein was extracted with 50 μl chloroform, and the digested DNA was transferred to the analysis vials.

Quantification was done by high-performance liquid chromatography with electrochemical and UV detection using a Prodigy octyldecyl silane column (particle diameter, 5 μm; pore size, 100 Å; Phenomenex, Torrance, CA) with a mobile phase of 3% acetonitrile–1 M NaOH–0.56% H_3PO_4 (pH 6). The samples were measured in duplicate, and each was injected twice.

8-oxodG was quantified in an electrochemical detector (Coulochem II; ESA model 5011 analytic cell; ESA, Chelmsford, MA), while dG was quantified by UV absorbance (LaChrom UV detector, model L-7400; Merck-Hitachi, Darmstadt, Germany). Peak areas were used for calculations.

RESULTS

MFs and mutation rates of GO mutants. Growth experiments in LB medium showed that inactivation of the genes in the GO system did not affect the growth rate, since similar doubling times were measured for PAO1 and the mutants (Table 2). Estimation of the spontaneous MFs revealed that the MF was elevated in these GO mutants. Compared to that of PAO1, the *mutT* mutant showed 28- and 140-fold, the *mutY* mutant 7.5- and 4-fold, and the *mutM* mutant 1.5- and 2-fold increases in the MF in response to rifampin and streptomycin, respectively. Each of these mutator phenotypes could be reverted by complementation with a plasmid containing the respective wild-type GO gene (Table 2).

Estimation of the mutation rates (probability of mutations/cell/generation) showed that both the *mutT* and the *mutY* mutant had significantly higher mutation rates than PAO1, while the *mutM* mutant did not (Table 2).

Increased development of resistance to antibiotics in GO mutants. To evaluate the capacity of the GO mutants to develop resistance to antibiotics, we identified the presence of resistant mutant subpopulations within the inhibition zones of antibiotics using disk diffusion (Fig. 1) and Etest strips and characterized their sizes by a ranking system described previously (35) (Table 2). We calculated the sizes of the resistant subpopulations by population analysis of GO mutants with respect to three different groups of antibiotics (β-lactams, fluoroquinolones, and aminoglycosides) (Fig. 2). The sizes of the resistant subpopulations correlated with the MFs of the three different mutants; these subpopulations were largest in the *mutT* mutant, smaller in the *mutY* mutant, and almost disappearing in the *mutM* mutant (Fig. 1 and 2; Table 2).

This demonstrates that even a moderate increase in the mutation rate has a measurable effect on the evolution of antibiotic resistance.

Mechanisms of resistance in GO mutants. To investigate which underlying mechanisms were responsible for the resistant subpopulations selected in the GO mutants, we picked up four resistant colonies of each subpopulation from the plates containing antibiotics. The MICs for the different colonies were measured in order to test for cross-resistance to other antibiotics, including tetracycline and chloramphenicol (Table 3).

The colonies isolated on ceftazidime had ≥9-fold-higher MICs than the respective mutants before the population analysis. This indicated increased β-lactamase activity, and we found that the basal level of β-lactamase was increased approximately fivefold, which might explain the elevated resistance to the β-lactam antibiotics.

The colonies selected on tobramycin showed a tendency toward higher tobramycin MICs than before exposure. The chloramphenicol MICs for the *mutY* and *mutT* mutants were

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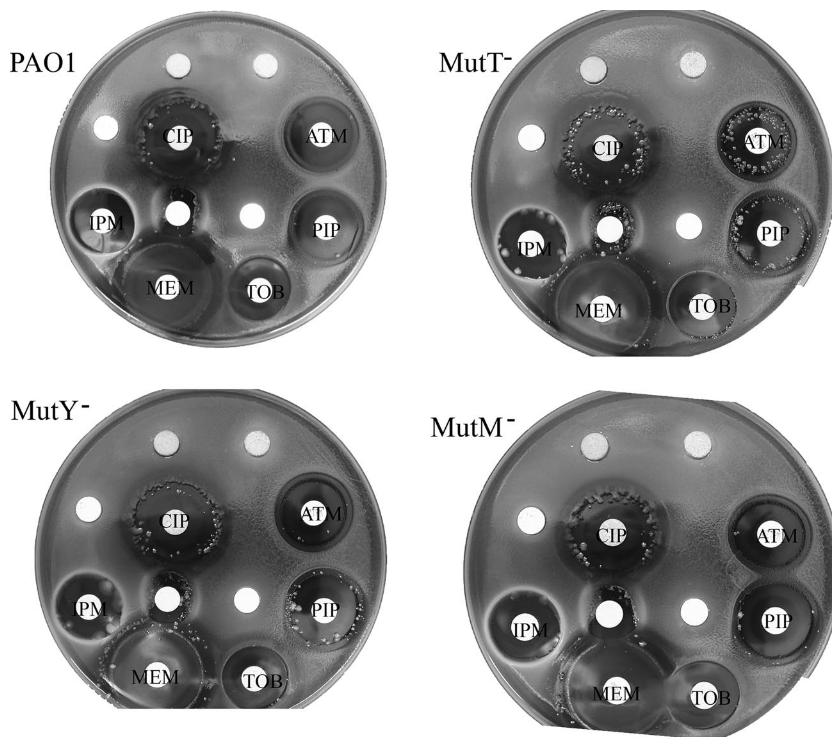

FIG. 1. Determination of susceptibilities of PAO1 and GO mutants (10^8 CFU) to antibiotics by disk diffusion. Colonies in the inhibition zones represent mutant subpopulations resistant to piperacillin (PIP), tobramycin (TOB), ciprofloxacin (CIP), aztreonam (ATM), meropenem (MEM), and imipenem (IPM).

two- and threefold higher than those determined before the population analysis, and resistant colonies in the inhibition zone of chloramphenicol were observed for all the resistant colonies selected on tobramycin.

The colonies isolated on ciprofloxacin-containing agar showed a three- to fourfold-decreased susceptibility to ciprofloxacin but expressed cross-resistance against tetracycline and chloramphenicol (MICs, > 256 μg/ml). The resistance against

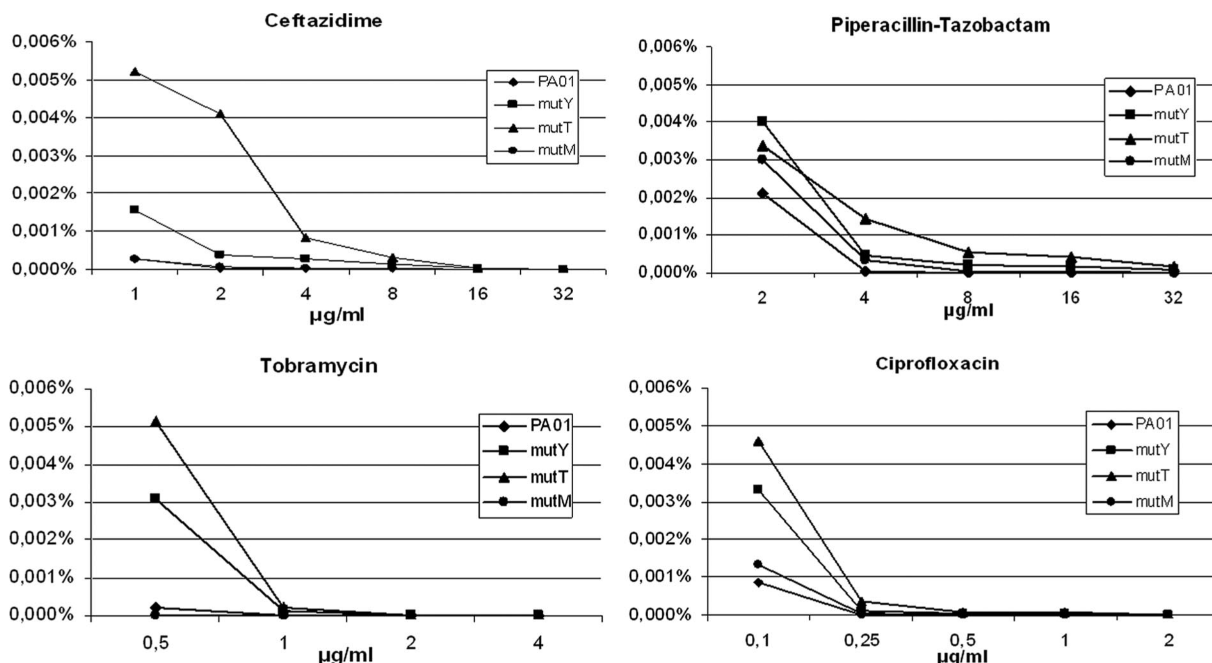

FIG. 2. Population analysis of the GO mutants in response to four different antibiotics: piperacillin-tazobactam, ceftazidime, tobramycin, and ciprofloxacin. The figure shows the percentage of the total bacterial population surviving as a function of antibiotic concentration.

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TABLE 3. Cross-resistance, increased β-lactamase activity, and expression of efflux pumps in resistant colonies[a]

Antibiotic used for selection[b] and strain	MIC (μg/ml)[c]					Fold change[d] in:					
						β-Lactamase activity[e]		Efflux pump expression			
	CAZ	CIP	TOB	CHL	TET	Basal	Induced	*mexF*	*mexD*	*mexB*	*mexY*
None, PAO1	2	0.19	1.5	32	4						
CAZ											
PAO1	20	0.125	1.75	ND	ND	4.4	1.07	ND	ND	ND	ND
mutT mutant	18	0.17	2	ND	ND	5.6	0.78	ND	ND	ND	ND
mutY mutant	26	0.13	2	ND	ND	4.9	0.88	ND	ND	ND	ND
mutM mutant	18	0.16	2	ND	ND	5.4	0.87	ND	ND	ND	ND
TOB											
PAO1	0.5	0.32	6	28 (+)	10	ND	ND	1.18	1.88	1.29	2.4
mutT mutant	0.69	0.16	3.75	86 (+)	5.5	ND	ND	1.04	0.86	0.89	0.76
mutY mutant	0.63	0.11	3.5	56 (+)	18	ND	ND	2.05	1.57	0.94	2
mutM mutant	0.38	0.08	0.9	20 (+)	4.25	ND	ND	ND	ND	ND	ND
CIP											
PAO1	0.44	0.75	1.06	>256	>256	ND	ND	1.25	618.73	5.5	1.77
mutT mutant	0.47	0.75	1.37	>256	>256	ND	ND	0.97	607.77	1.07	1.04
mutY mutant	0.44	0.88	1.19	>256	>256	ND	ND	0.75	437.72	0.59	0.89
mutM mutant	0.44	0.75	1.25	>256	>256	ND	ND	11.3	342.16	0.73	5.7

[a] From the population analysis of the GO gene (*mutT*, *mutY*, and *mutM*) mutants isolated after selection on ceftazidime, tobramycin, or ciprofloxacin.
[b] CAZ, ceftazidime; TOB, tobramycin; CIP, ciprofloxacin.
[c] Each MIC is the mean for four colonies (two successive determinations per colony), except for PAO1 after selection on tobramycin, where the MIC is the mean for two colonies. CHL, chloramphenicol; TET, tetracycline; ND, not detected. Plus signs in parentheses indicate that the colonies isolated from LB agar plates containing tobramycin showed a small subpopulation resistant to chloramphenicol.
[d] Relative to the respective nonresistant strain.
[e] The induced β-lactamase activity of PAO1 was 26.5-fold higher than its basal β-lactamase activity.

these antibiotics indicates an upregulation of efflux pumps (32, 37).

To investigate whether this was the case, the expression of the *mexB*, *mexD*, *mexY*, and *mexF* genes, encoding different efflux pumps, was determined by real-time PCR for two of the resistant colonies. The *mexD* gene was hyperexpressed in the ciprofloxacin-resistant isolates (Table 3). The sequence of the *nfxB* regulatory gene showed that all the ciprofloxacin-resistant colonies had mutations in this transcriptional regulator of MexCD-OprJ. The base changes in *nfxB* found in the MutY mutant correspond to the expected mutation, a G · C → T · A transversion creating premature stop codons at positions 91 and 332. The *nfxB* mutation localized in the MutM mutant at position 346 was a G · C → T · A transversion creating a premature stop codon, but the mutation at position 113 was an insertion leading to a frameshift. This frameshift mutation is highly unlikely to be a consequence of *mutM* inactivation. The base changes found in the *nfxB* gene in the MutT mutant are A · T → C · G transversions (positions 41 and 528) causing an amino acid change. This base change could be explained by the hypothesis that 8-oxodG mispairs with adenine and A · T becomes A · 8-oxodG (42). We also found *nfxB* mutations in the PAO1 mutants generating stop codons at position 67 and 347.

The tobramycin-resistant mutants with increased resistance to chloramphenicol did not show upregulation of any of the efflux pumps investigated; therefore, the data suggest that other resistance mechanisms, such as drug-modifying enzymes or alterations in the lipopolysaccharide or outer membrane proteins, may be involved (36).

DNA oxidative lesion (8-oxodG) measurements in GO mutants. To investigate the role of inactivation of the GO system in the level of DNA oxidation, we measured the levels of 8-oxodG per 10^6 dG molecules and found that they were higher in all three GO mutants (3.13-, 3.43-, and 2.12-fold higher in the *mutT*, *mutY*, and *mutM* mutants, respectively) than in the reference strain PAO1. This indicates a significant association between a deficiency in oxidative repair and the occurrence of oxidative DNA lesions.

Exposure to PMNs increases the MF. To analyze the effect of oxidative stress on these mutants that are unable to repair their DNA oxidative damage, we exposed the GO mutants to activated PMNs. The oxidative stress response from the activated PMNs increased the MF in response to rifampin 73-fold for the *mutT* mutant, 57-fold for the *mutY* mutant, and 0.34-fold for the *mutM* mutant over that for PAO1. This suggests that the frequency of spontaneous mutations was increased after exposure to the ROS liberated from the PMNs.

To investigate the role of inactivation of the GO system in the level of DNA oxidation, we measured the levels of 8-oxodG. After exposure to activated PMNs, the level of 8-oxodG per 10^6 dG molecules was increased 2.58-fold for the *mutT* mutant, 1.85-fold for the *mutY* mutant, and 1.19-fold for the *mutM* mutant over that for PAO1. The emergence of DNA oxidative lesions depends on the ROS in the environment as well as on the capacity to protect against and repair mutations.

Occurrence of *P. aeruginosa* isolates from CF patients with oxidative repair deficiencies. Two of the 11 *P. aeruginosa* CF isolates with a hypermutable phenotype and high levels of

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8-oxodG showed loss-of-function mutations in the oxidative repair genes. This is the first time that mutations in the GO system have been reported in natural niches. A clinical CF isolate, 73419G, had an insertion of G at codon 53 of *mutT*, creating a premature stop codon. Complementation of this clinical *mutT* mutant with a plasmid containing the wild-type gene led to a decrease in the MF from $2.08e10^{-7}$ to $2.68e10^{-8}$ (eightfold) on rifampin as well as an increased susceptibility to tobramycin, confirming that this mutation led to a nonfunctional MutT enzyme (data not shown). Another clinical CF isolate, 66999E, had mutations generating stop codons in both *mutY* (amino acid [aa] 305) and *mutL* (aa 591). Complementation with wild-type *mutY* lowered the MF from $5.31e10^{-7}$ to $5.01e10^{-8}$ (10-fold) on streptomycin and resulted in a reduction in the resistant subpopulation after exposure to piperacillin, tobramycin, or aztreonam (data not shown). Surprisingly, complementation with wild-type *mutL* had no influence on the MF, but the complemented strain became even more susceptible to antibiotics than the complemented *mutY* mutant.

DISCUSSION

The genetic background of *P. aeruginosa* mutators from CF patients has been shown to be mutations in the DNA MMR system. However, Hogardt et al. suggested that not all clinical CF isolates with the hypermutable phenotype can be related to a dysfunction of MutS and that the mutator phenotype may just as well be linked to defects in other DNA repair proteins (19). Various studies have shown that both *mutL* and *uvrD* from the MMR system also can give rise to the HP phenotype (45, 47). Oliver et al. (48) found indications of the involvement of mutations in genes belonging to the DNA oxidative repair system, such as *mutY*, in the lack of PCR amplification of this gene in some HP clinical isolates. In this work we provide evidence that mutation in the GO system is also a mechanism leading to hypermutation. We showed that, indeed, inactivation of the *mutT*, *mutY*, and *mutM* genes, involved in the GO system, led to elevated MFs, which correlated with an increased frequency of development of resistance to antibiotics. The results presented here demonstrate that impairment of the GO system might contribute to improved fitness of these mutants in the lungs of CF patients. Comparison of the effects of inactivation of the enzymes of the GO system to those for the enzymes of the MMR system showed that the MF is differentially influenced by the two DNA repair systems. While HP isolates with defects in the MMR enzymes are classified as strong mutators, isolates with defects in the GO system are weak mutators. It has been proposed that the MF has to be at least 20-fold higher than the MF of PAO1 in order for a *P. aeruginosa* isolate to be defined as an HP (40), and according to this definition, only the *mutT* mutant expressed an HP phenotype. The weak mutator phenotype has been found in *E. coli* isolates (23%) from a variety of sources and to a lesser extent in *P. aeruginosa* isolates (6%), but no clear definition of a weak mutator is available. In this study we considered isolates for which the MF increased <5-fold over that for PAO1 to be weak mutators and isolates for which the MF increased 5- to 20-fold to be moderate mutators. The absence of one of the enzymes from the *P. aeruginosa* GO system does not have as great an effect on the MF as that described for *E. coli* (13). When the effects of inactivation of the three enzymes from the GO system of *P. aeruginosa* on the MF were compared, a graduated effect could be observed: the *mutT* mutant was the strongest mutator, the *mutY* mutant was a moderate mutator, and the *mutM* mutant was a weak mutator. This is in accordance with findings reported for *E. coli* (13, 41, 61). However, we have not investigated the occurrence in *rpoB* (responsible for rifampin resistance) of specific G · C → T · A or A · T → C · G mutations in GO mutants, and therefore we might have underestimated the MF. It has been shown that the weak mutator phenotype can drive bacterial evolution under selection pressure (3, 29, 50). However, the genetic background of the weak mutators has not been elucidated. Our data showed that inactivation of the GO system might be one of the mechanisms involved in the occurrence of the moderate and weak mutator phenotypes. The increase in the frequency of spontaneous mutations in strains with nonfunctional GO systems is probably due to the accumulation of unrepaired oxidative DNA lesions. Measurements of the 8-oxodG levels in these mutants showed higher levels of this mutagenic DNA lesion in GO mutants than in PAO1.

In the lungs of CF patients, the chronic infection with *P. aeruginosa* is characterized by activated PMNs, which are an important source of mutagenic ROS (25). After exposure to activated PMNs, >50-fold increases in the MF were observed in *mutT* and *mutY* mutants. This suggests that in vivo, ROS can lead to important increases in the MF for GO mutants. We also showed that the increase in the MF after PMN exposure was associated with a moderate increase in the levels of 8-oxodG lesions of the DNA, suggesting that the unrepaired oxidative DNA lesions accumulate in the GO mutants. The discrepancy between the large increase in the MF after PMN exposure and the moderate increase in the levels of 8-oxodG might be due to the occurrence of other mutagenic DNA oxidative lesions that were not measured in this study.

The correlation between increased MFs of isolates and the development of resistance to antibiotics has been demonstrated repeatedly (3, 35, 50). Our data show that the increased MF due to the lack of function of the MutT, MutY, and MutM enzymes led to increased development of resistance to antibiotics. When exposed to antibiotics, the GO mutants showed larger resistant subpopulations than controls, demonstrating that they had an advantage in survival in an environment with selective pressure. These results suggests that the MF does not have to be as high as 10^{-6} (as for the MMR mutants, especially *mutS* mutants) to influence the adaptation to antibiotics. Even a small increase in mutation rates can significantly influence the rate of adaptation. Despite the fact that most mutations are neutral or deleterious, the weak mutators have increased possibilities of favorable mutations, and their evolution rates are accelerated (3). The presence of a mutator strain is a risk factor in the treatment of infectious diseases, since such strains might be responsible for therapeutic failures (10). To date there is no obvious strategy for eliminating or reversing mutator phenotypes in bacterial populations. A clinical strain with a mutant subpopulation resistant to a certain antibiotic should be treated with the concentration of drug that eliminates the subpopulation to clear the infection, the mutation prevention concentration (11).

Our study showed that the development of resistance to

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antibiotics by GO mutants occurred through mechanisms similar to those described for *P. aeruginosa mutS* mutants. Resistance to β-lactam antibiotics was caused by increased β-lactamase expression, and resistance to fluoroquinolones was due to increased activity of efflux pumps, such as MexCD-OprJ. In accordance with previous studies, overproduction of MexCD-OprJ was caused by mutations in the pump regulator *nfxB* (26, 27, 51). Similar mechanisms have been demonstrated to occur in multiresistant HP *P. aeruginosa* isolates from CF patients (17, 52).

We suggest that beyond the MMR mutants, which are favored in CF, the weak, moderate, and strong mutators due to mutations in the GO repair system are also favored in the CF lung, because these mutants will accumulate beneficial mutations more rapidly, increasing the phenotypic variation necessary to overcome the immune response and antibiotic treatment. However, in HP isolates, most mutations are deleterious, and being a strong mutator also has some cost for long-term adaptation, such as reducing fitness in secondary environments (15). Once a mutator population is well adapted to a niche, the load of deleterious mutations lowers the fitness of the population and thereby favors a low (or lower) mutation rate (60).

Complementation and sequence analysis of clinical HP resistant isolates showed that mutations in the MMR system are the most frequently encountered mechanism of HP and *mutS* is the most affected gene (47). We found CF isolates with mutations in genes involved in the GO repair system. We report for the first time a mutator strain from a natural CF population with an inactivation of *mutT* due to an insertion resulting in a premature stop codon. The insertion was found within the first 130 aa from the N terminus, in a region that has been shown to be 38% identical to *E. coli mutT* and is a highly conserved residue with 8-oxodG hydrolase activity (49). There is also evidence that *E. coli* has various MutT-type proteins contributing to or backing up the MutT function (23, 28), and there are predictions that *P. putida* and *P. aeruginosa* could also have homologous back-up proteins (54). If these proteins also exist in *P. aeruginosa*, they, too, could influence the MF and the development of resistance.

We also identified *P. aeruginosa* CF isolates defective in *mutY*, but in combination with mutations in MMR genes. Complementation of the disrupted gene with the wild-type gene from PAO1 reduced the GO mutants' MFs and increased their susceptibilities to antibiotics. This confirms the notion that the mutated genes encode for nonfunctional proteins. These results support the hypothesis that GO mutants might also be selected during exposure to high selective pressure from antibiotics and oxidative stress found in the lungs of CF patients.

The GO mutants are not able to repair the oxidative DNA lesions caused by the increased oxidative stress present in the lungs of CF patients; consequently, the MFs of these mutant *P. aeruginosa* isolates increase. The increased MF confers an adaptive advantage, facilitating the development of resistance to antibiotics. This suggests the possible use of antioxidants in the treatment of CF patients, or patients with other chronic bacterial infections, in order to prevent bacterial DNA oxidative lesions and to prevent the emergence of antibiotic resistance. Studies supporting this hypothesis are in progress in our laboratory.

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