

Sublethal Ciprofloxacin Treatment Leads to Rapid Development of High-Level Ciprofloxacin Resistance during Long-Term Experimental Evolution of *Pseudomonas aeruginosa*

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The dynamics of occurrence and the genetic basis of ciprofloxacin resistance were studied in a long-term evolution experiment (940 generations) in wild-type, reference strain (PAO1) and hypermutable (PAO Δ mutS and PAOMY-Mgm) *P. aeruginosa* populations continuously exposed to sub-MICs (1/4) of ciprofloxacin. A rapid occurrence of ciprofloxacin-resistant mutants (MIC of ≥ 12 μ g/ml, representing 100 times the MIC of the original population) were observed in all ciprofloxacin-exposed lineages of PAO Δ mutS and PAOMY-Mgm populations after 100 and 170 generations, respectively, and in one of the PAO1 lineages after 240 generations. The genetic basis of resistance was mutations in *gyrA* (C248T and G259T) and *gyrB* (C1397A). Cross-resistance to beta-lactam antibiotics was observed in the bacterial populations that evolved during exposure to sublethal concentrations of ciprofloxacin. Our study shows that mutants with high-level ciprofloxacin resistance are selected in *P. aeruginosa* bacterial populations exposed to sub-MICs of ciprofloxacin. This can have implications for the long-term persistence of resistant bacteria and spread of antibiotic resistance by exposure of commensal bacterial flora to low antibiotic concentrations.

In the airways of patients with cystic fibrosis (CF), antibiotic treatment is an important selective pressure that influences the adaptation and evolution of *Pseudomonas aeruginosa*. As a result of this adaptation, the bacteria persist for long periods of time, undergoing up to 200,000 generations in the CF lung (1). Patterns of parallel evolution at the levels of phenotype, gene expression, and genotype have been documented in CF, indicating repeatable patterns of long-term adaptation.

One of the common evolutionary traits is the development of antibiotic resistance, which is based on genetic variation and the selection of genotypes that this generates. *P. aeruginosa* strains with a hypermutator phenotype (conferring increased rates of mutation) are frequently encountered in bacterial populations from the CF lung (2, 3), and acquisition of this phenotype will increase the chances of acquiring antibiotic resistance by mutational events.

Exposure of *P. aeruginosa* to ciprofloxacin, which is a DNA-damaging antibiotic, stimulates bacteria to produce reactive oxygen species (ROS) that can directly damage DNA and lead to accumulation of mutations (4). The role of ciprofloxacin at inhibitory concentrations in development of resistance has been well established in bacterial populations with normal or increased mutation frequencies (5) and in evolution experiments (6, 7). In clinical settings, however, compartments where ciprofloxacin does not reach bacterial MIC are created, such as inside the sputum in the conductive zones of the airways (8). The anaerobic conditions inside the thick CF mucus might also decrease the efficacy of quinolones (9), requiring higher local concentrations. In addition, ciprofloxacin is excreted in sweat and present on the skin at low concentrations for long periods of time (10), with rapid development of resistance in skin bacteria. Epidemiological studies have shown an association between the use of quinolones and the occurrence of methicillin-resistant *Staphylococcus aureus* (11).

The role of long-term exposure of *P. aeruginosa* to sub-MICs of ciprofloxacin for the development of resistance has not been in-

vestigated before but is clinically relevant and can reveal important bacterial adaptive pathways. It has been shown in *Escherichia coli* that sublethal levels of bactericidal antibiotics induce mutagenesis that can lead to multidrug resistance (12). Recently, it has been shown in *E. coli* that very low antibiotic concentration can select *de novo* mutants, which would take over in a susceptible population (13). Long-term evolution experiments with microorganisms have been used to study the dynamics of evolutionary adaptation (14).

Therefore, we decided to investigate the evolution of *P. aeruginosa* during many (more than 900) bacterial generations exposed to sublethal concentrations of ciprofloxacin and to compare it to the evolution without selective pressure, focusing on the development of resistance to ciprofloxacin. To investigate the role of the mutation rates in the dynamics of bacterial evolution under the described conditions, two mutator strains due to mutations in DNA mismatch repair and oxidative damage repair were employed, along with the reference strain PAO1. Both types of mutators have been described in CF patients (2, 15, 16).

MATERIALS AND METHODS

Bacterial strains. Three *P. aeruginosa* strains were used for this study: the wild-type PAO1 (mutation rate, 6.75×10^{-9}) and the two mutator strains

Received 12 March 2013 Returned for modification 29 March 2013

Accepted 12 June 2013

Published ahead of print 17 June 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00493-13>.

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doi:10.1128/AAC.00493-13

PAO1ΔmutS and PAOMY-Mgm (17). PAO1ΔmutS is deficient in the DNA mismatch repair system (mutation rate, 5×10^{-6}), and the PAOMY-Mgm mutant is unable to repair oxidative DNA damage (mutation rate, 1.9×10^{-7}) (17).

Experimental setup of the long-term evolution study. The three strains were tested in triplicates (lineages A, B, and C) with or without 0.05 μg/ml ciprofloxacin, representing 1/4 the MIC of ciprofloxacin (0.19 μg/ml), which increases the doubling time from 24.5 (± 1.94) min to 32 (± 1.25) min. For each strain, a single colony was inoculated in Luria-Bertani (LB) medium and grown overnight at 37°C. One hundred microliters of each starter culture was inoculated into six different flasks each with 100 ml fresh preheated LB medium, allowing the growth of approximately 10 generations/day (10^6 CFU/ml starting inoculum to 10^9 CFU/ml ending inoculum). Ciprofloxacin (0.05 μg/ml) was added to three of the six cultures. The cultures were propagated daily for 94 days, allowing for triplicates to grow with and without addition of 0.05 μg/ml ciprofloxacin. The ciprofloxacin concentration remained the same, and a steady CFU count was maintained throughout the study. Daily streaking of the overnight cultures on 5% blood agar plates (5% horse blood; SSI Diagnostica, Hillerød, Denmark) was performed to check for contamination, and the incubated cultures were stored for two nights at 4°C to restart the experiment in case of contamination. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectrometry was applied to confirm the identity of the strains as *Pseudomonas aeruginosa* at days 73, 80, 87, and 94. Three colonies representative of each strain were selected from the CFU plate at the 10^{-7} dilution and subjected to MALDI-TOF analysis. To confirm that the three *P. aeruginosa* lineages (A, B, and C) belonged to PAO1, PAO1ΔmutS, or PAOMY-Mgm, respectively, confirmatory PCR was performed.

Bacterial population analysis. To characterize the evolution of the bacterial populations grown for 940 generations in the presence or absence of subinhibitory concentrations of ciprofloxacin (1/4 the MIC), bacterial populations of PAO1, PAO1ΔmutS, and PAOMY-Mgm at different generations (days 3, 10, 17, 24, and 94) were tested for susceptibility to ciprofloxacin. For each of the three lineages of the three strains, a large inoculum (10^8 CFU/ml; a 10^{-1} dilution of an overnight culture) was plated on blood agar plates, and an Etest strip of ciprofloxacin was applied. The concentration of ciprofloxacin on the Etest that inhibited the bacterial population was defined as the “population MIC.” To distinguish between different sizes of the resistant subpopulations that grew in the inhibition zone (Fig. 1), four descriptive entities were defined: (i) “double zone” if the resistant colonies exhibited confluent growth, (ii) +++ if the number of colonies was higher than 100, (iii) ++ if the number of colonies was between 10 and 100, and (iv) + if fewer than 10 colonies were present. Figure 1 shows an example of the bacterial population analysis. Colonies grown in the inhibition zone were harvested for further analysis.

Antibiotic susceptibility. Ciprofloxacin-resistant subpopulations (+, ++, or ++++) from days 3, 10, 17, 24, and 94 of the three lineages of each strain evolved in the presence or absence of ciprofloxacin were harvested by scraping from the inhibition zone and retested for ciprofloxacin susceptibility after 2 passages in antibiotic-free medium to confirm the mutagenic nature of their phenotype. To investigate the homogeneity of the harvested resistant subpopulations, antibiotic susceptibility and sequence variability of quinolone resistance determinants were determined for 6 to 9 independent single resistant colonies for each of the three strains.

Sequencing of the quinolone resistance determinants (*nfxB*, *gyrA*, *gyrB*, and *parC*) on all these resistant subpopulations (a total of 127 colonies) was performed. The MIC of ciprofloxacin for the selected ciprofloxacin-resistant subpopulations was determined by the dilution method in microtiter plates as previously described (18). To determine the occurrence of cross-resistance, the MICs of ciprofloxacin, meropenem, ceftazidime, and tobramycin against each of the bacterial populations evolved in the presence and absence of ciprofloxacin were investigated by Etest according to the instructions of the manufacturer (inoculum, 10^5 CFU/ml).

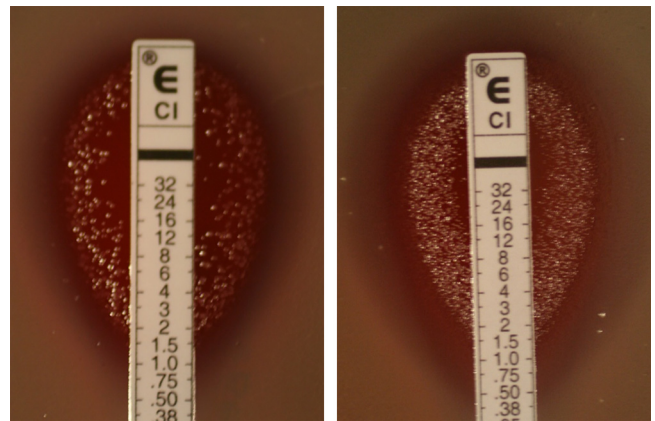


FIG 1 Characterization of the susceptibility to ciprofloxacin of the bacterial population and assignment of population MIC values. To distinguish between different sizes of the resistant subpopulations that grew in the inhibition zone, four descriptive entities were defined: (i) “double zone” if the resistant colonies exhibit a confluent growth, (ii) +++ if the number of colonies was higher than 100, (iii) ++ if the number of colonies was between 10 and 100, and (iv) + if fewer than 10 colonies were present. The descriptions of the two bacterial populations in this figure are thus as follows: in the left panel, the population MIC is 1.5 μg/ml, there is no double zone (dz−), and the size of the resistant subpopulation is ++++; in the right panel, the population MIC is 1.5 μg/ml, a double zone is present until 12 μg/ml (dz 12), and resistant colonies in the inhibition zone are also present (+).

Formation of reactive oxygen species under treatment with 0.05 μg/ml ciprofloxacin. As the bactericidal effect of ciprofloxacin has been shown to be hydroxyl radical dependent (4), we wanted to test if the sub-MIC level of ciprofloxacin used in our study led to formation of oxygen radicals. This was tested as previously described (4). In short, exponentially grown *P. aeruginosa* was exposed to 0.05 μg/ml ciprofloxacin and 5 μM hydroxyl radical indicator 3'-(*p*-hydroxyphenyl)fluorescein (HPF) for 3 h. To confirm the specificity of the assay, thiourea (150 mM) (Sigma) was used as a scavenger of hydroxyl radicals. After incubation, hydroxyl radical formation was measured in a FACScanto flow cytometer (BD Biosciences, San Jose, CA) with a 488-nm argon laser and a 530/30-nm band pass emission filter for recording of HPF fluorescence as the area of the pulse in FL1. To maximize resolution, samples were analyzed at low flow rate corresponding to 10 μl/min. A least 10,000 events were recorded for each sample. The following photomultiplier tube (PMT) voltage settings were used for collection of linear amplified signals: 200 (forward scatter [FSC]), 420 (side scatter [SSC]), and 550 (FL1). Cytometer Setup and Tracking Beads (BD) were used for instrument calibration. The HPF fluorescence intensity is expressed as the mean channel in FL1-A.

Measurements of mutation frequency in *P. aeruginosa* mutant isolates. The mutation frequencies were investigated on LB plates containing rifampin (300 mg/liter) and streptomycin (500 mg/liter) as previously described (3). An isolate was considered hypermutable when the mutation frequency was 20-fold higher than the mutation frequency of the reference strain PAO1 ($\geq 3 \times 10^{-7}$).

Sequencing of quinolone resistance-determining regions (QRDRs). Sequencing of ciprofloxacin resistance-determining genes *nfxB*, *gyrA*, *gyrB*, *parC*, and *parE* was performed on purified total DNA (Qiagen) of the selected resistant isolates and of the starting bacterial strains (PAO1_start, mutS_start, and mutMY_start). The genes were amplified by PCR using previously described primers (17). The obtained sequences were compared to the sequence of the reference strain PAO1 by applying DNASIS Max version 2.0 (Hitachi Software Engineering).

Fitness cost analysis. To investigate if the type of mutations in *gyrA* and *gyrB* has a fitness cost for the growth of the mutants, growth curves

(optical density at 600 nm [OD₆₀₀]) were constructed in LB and LB with 0.05 µg/ml ciprofloxacin, and the doubling time was calculated on the exponential phase of the curve.

Nutritional status determination. The nutritional status of the evolved populations was determined by spreading a 10⁻⁶ dilution of an overnight culture on “blue” plates. After incubation at 37°C overnight, 50 colonies/lineage were replica plated to both glucose minimal medium and LB. Possible auxotrophs were scored as cells forming colonies on the LB plates but not on the minimal medium plates after 48 h of incubation at 37°C.

RESULTS

Selection of preexisting resistant subpopulations occurs during exposure to sub-MICs of ciprofloxacin. Analysis of the bacterial populations of PAO1, PAOΔmutS, and PAOMY-Mgm showed a gradual increase in the size of resistant subpopulations during 940 generations in the lineages treated with a sublethal concentration of ciprofloxacin (1/4 the MIC), while no increase in resistance was observed for the populations that evolved in the absence of selective pressure (Fig. 2). It is important to underline that at this concentration the bacteria are not killed but grow slightly slower (the doubling time increases from 24.5 to 32 min).

The resistant mutants occurred and were enriched faster in the mutator populations than in PAO1, as seen in Fig. 2 and Table S1 in the supplemental material. The fastest occurrence of resistance and the highest MIC were observed in PAOΔmutS, followed by PAOMY-Mgm and PAO1. This shows an association between the mutation rate of the strain and the size and speed of the occurrence of the resistant subpopulation (Fig. 2).

Resistant subpopulations creating double zones on Etests inhibited by 12 µg/ml ciprofloxacin (100 times the MIC) were observed in all ciprofloxacin-exposed lineages of PAOΔmutS and PAOMY-Mgm after 100 and 170 generations, respectively, and in one of the PAO1 lineages after 240 generations (see “MIC and susceptibility of population” in Table S1 in the supplemental material). No resistant subpopulations were observed in the starting population of PAO1, showing that the ciprofloxacin-resistant mutants that occurred in the three PAO1 lineages during ciprofloxacin exposure were present in a very small proportion in the starting wild-type population or occurred *de novo*. In contrast, resistant colonies were observed in the starting populations of the two mutator strains (PAOΔmutS and PAOMY-Mgm), showing the preexistence of antibiotic-resistant mutants in these bacterial populations (see Tables S1 and S2 in the supplemental material).

Type of mutations responsible for high-level ciprofloxacin resistance. In all three lineages of PAOΔmutS, the *gyrA* C248T mutants leading to T83I were selected by 1/4 the MIC of ciprofloxacin during the evolution experiment. The MICs of the resistant colonies to ciprofloxacin increased from 3 and 4 µg/ml in the starting population to 64 µg/ml at day 3 and 128 µg/ml at day 94 (see Table S1 in the supplemental material).

This *gyrA* mutation is in accordance with the type of mutations (transitions and frameshifts) encountered in mutators with an impaired mismatch repair (MMR) system (19). The same mutation was found in resistant PAOΔmutS isolates of control populations after 24 days of passage in LB medium (see Table S1b in the supplemental material) and in single resistant colonies of the initial population, confirming the selection of the preexisting mutation in the original population (see Table S2 in the supplemental material).

The ciprofloxacin-resistant mutants which emerged already at day 3 in the PAOMY-Mgm population exposed to ciprofloxacin

were caused by a G-to-T transversion at position 259 in *gyrA* in two of the lineages (A and B) and by a C-to-A transversion at position 1397 in *gyrB* in the third lineage (C) (Table 1). The same *gyrA* mutation was found in ciprofloxacin-resistant colonies from the control populations at day 94, suggesting that this mutation was preexisting in the original PAOMY-Mgm population.

Transversions are the specific type of mutations to be expected in mutants of the DNA oxidative repair system (GO) due to mispairing with the uncorrected oxidative lesion of guanine, 8-oxo-2'-deoxyguanosine (8oxodG). The ciprofloxacin MICs of the preexisting resistant mutants in the starting population were 4 µg/ml and 6 µg/ml (see Table S2 in the supplemental material) and increased to 16 µg/ml at day 3 and 32 µg/ml at day 94 during the evolution experiment (Table 1; see Table S1 in the supplemental material).

The fitness cost analysis of the three types of mutations identified in our study to cause ciprofloxacin resistance, i.e., C248T (T83I) and G259T (D87Y) in *gyrA* and C1397A (S465Y) in *gyrB*, showed that these mutations did not reduce the growth rate (no fitness cost) compared to that of the starting strains (Table 2). The occurrence of these mutations restored the slightly lower growth rates of the starting strains observed in the presence of the 1/4 the MIC of ciprofloxacin.

gyrA parC double mutants (*gyrA*, C248T; *parC*, C260T) were identified only in the PAOΔmutS A and C lineages. The mutation in *parC* leading to replacement of Ser 87 with Leu occurred at a late stage during the evolutionary experiment (940 generations), confirming that *parC* mutations are second-step mutations. A *gyrA gyrB* double mutant was also identified at day 94 in PAO1 lineage C (Table 1), which presented only a *gyrB* mutation until day 87 (data not shown), showing the late occurrence of double mutations during the evolution study.

Fitness in secondary environments. To test whether adaptation in the rich LB medium resulted in the loss of metabolic fitness in secondary environments, we determined the frequency of possible auxotrophs (lack of growth on glucose minimal media) in all the populations that evolved in the presence or absence of ciprofloxacin at days 24 and 94 of the long-term experiment. We found an increased proportion of colonies with lack of growth on minimal medium in mutator, compared to nonmutator, lineages (see Table S4 in the supplemental material).

Reactive oxygen species are produced during treatment with sub-MICs of ciprofloxacin. Measurement of reactive oxygen species under treatment with 0.05 µg/ml ciprofloxacin showed a 10-fold increase in the level of hydroxyl radicals (FL1-A mean, 2,564) compared to that in untreated bacterial cells (FL1-A mean, 233). No fluorescence was measured when thiourea, which is a hydroxyl radical scavenger, was added (data not shown).

No changes in the mutation frequencies of the bacterial populations of the three strains after evolution in the presence of sublethal concentrations of ciprofloxacin were observed.

Cross-resistance to beta-lactam antibiotics after exposure to sub-MICs of ciprofloxacin. Selection of subpopulations with decreased susceptibility to the beta-lactam antibiotics ceftazidime and meropenem was observed in the bacterial populations that evolved during exposure to sublethal concentrations of ciprofloxacin (see Table S3 in the supplemental material). This was in contrast to the evolution in the absence of ciprofloxacin, where no increase in resistance was observed, suggesting that the changes in the susceptibility to beta-lactams are related to the ciprofloxacin treatment. For example, for PAO1, ceftazidime- and meropenem-

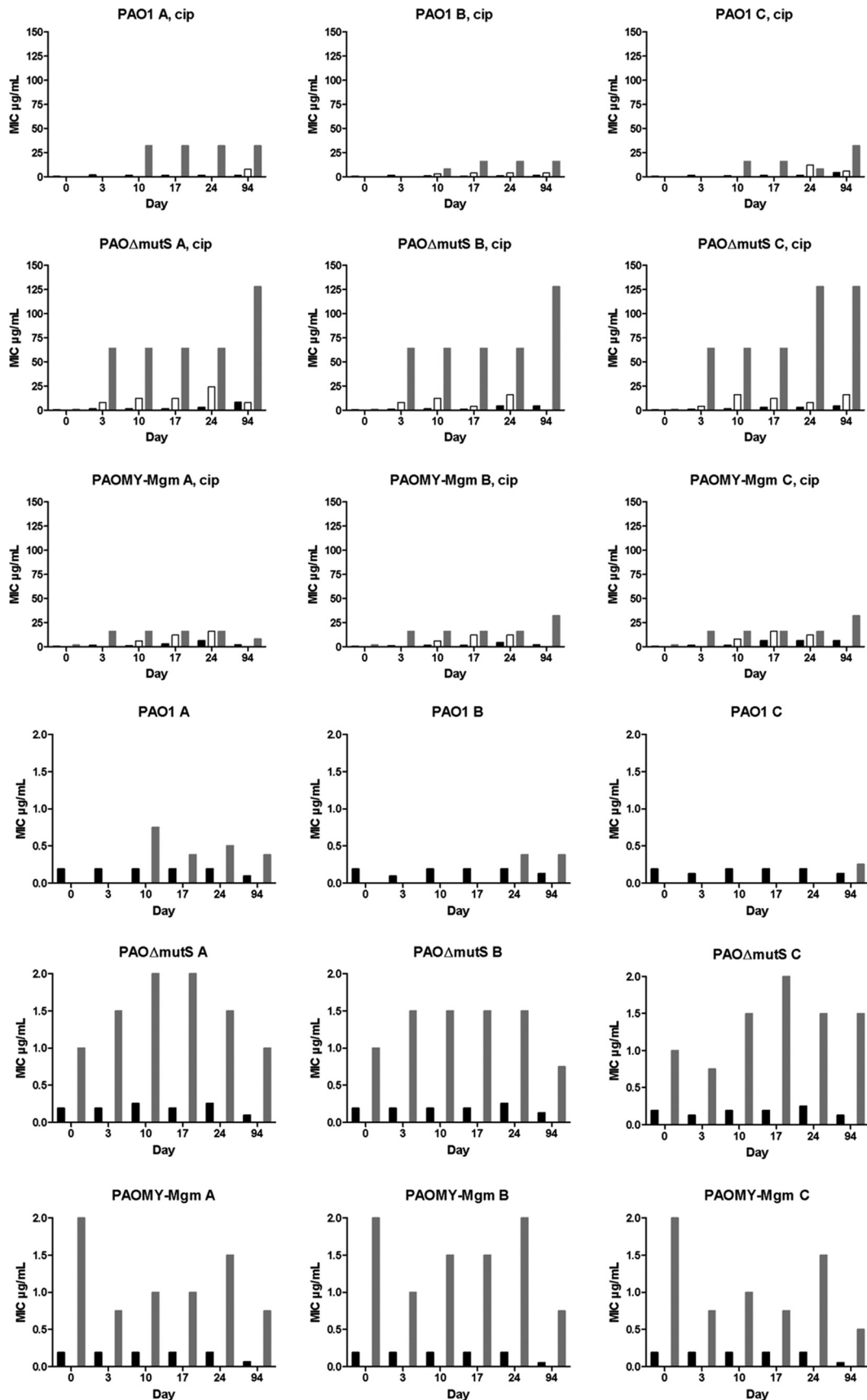


FIG 2 Development of resistance to ciprofloxacin in ciprofloxacin-treated (top three rows) and untreated (bottom three rows) PAO1, PAO Δ mutS, and PAOMY-Mgm during the evolution experiment. Resistant mutants occurred faster in the mutator populations than in PAO1, with an especially drastic increase in MICs for the ciprofloxacin-treated PAO Δ mutS strains. Black bars, MICs of population; white bars, MICs of double zone; gray bars, MICs of resistant colonies. Notice the difference in the numerical size of the ordinates in the top three and bottom three rows.

TABLE 1 Overview of mutations found in quinolone resistance-determining regions (*nfxB*, *gyrA*, *gyrB*, and *parC*) in ciprofloxacin-resistant subpopulations selected at days 3, 10, 24, and 94 of the evolution experiment in the three lineages of PAO1, PAOΔmutS, and PAOMY-Mgm under exposure to 1/4 the MIC of ciprofloxacin^a

Lineage (MIC [μg/ml] on days 3, 10, 17, 24, 94)	Gene	Mutation on day:				
		3	10	17	24	94
PAO1-A-cip (ND, 32, 32, 32, 32)	<i>nfxB</i>	—	Del C pos 6	—	—	—
	<i>gyrA</i>	—	C248T	C248T	C248T	C248T
	<i>gyrB</i>	—	—	—	—	—
	<i>parC</i>	—	—	—	—	—
PAO1-B-cip (ND, 8, 16, 16, 16)	<i>nfxB</i>	—	—	Del C pos 6	G436A	—
	<i>gyrA</i>	—	G259T	G259T	G259T	G259T
	<i>gyrB</i>	—	—	—	—	—
	<i>parC</i>	—	—	—	—	—
PAO1-C-cip (ND, 16, 16, 8, 32)	<i>nfxB</i>	—	336 ins G	G461A	G461A	—
	<i>gyrA</i>	—	—	—	—	G259T
	<i>gyrB</i>	—	C1397A	C1397A	C1397A	C1397A
	<i>parC</i>	—	—	—	—	—
PAOΔmutS-A-cip (64, 64, 64, 64, 128)	<i>nfxB</i>	C434T	—	—	G461A	—
	<i>gyrA</i>	C248T	C248T	C248T	C248T	C248T
	<i>gyrB</i>	—	—	—	—	—
	<i>parC</i>	—	—	—	—	C260T
PAOΔmutS-B-cip (64, 64–128, 64, 64, 128)	<i>nfxB</i>	—	G538A	G538A	—	—
	<i>gyrA</i>	C248T	C248T	C248T	C248T	C248T
	<i>gyrB</i>	—	—	—	—	—
	<i>parC</i>	—	—	—	—	—
PAOΔmutS-C-cip (64, 64, 64, 64, 128)	<i>nfxB</i>	—	G461A	—	—	T499C
	<i>gyrA</i>	C248T	C248T	C248T	C248T	C248T
	<i>gyrB</i>	—	—	—	—	—
	<i>parC</i>	—	—	—	—	C260T
PAOMY-Mgm-A-cip (16, 16, 16, 16, 8)	<i>nfxB</i>	C473A	—	G538T	G539T	G140T
	<i>gyrA</i>	—	—	—	ND	—
	<i>gyrB</i>	C1397A	C1397A	C1397A	ND	C1397A
	<i>parC</i>	—	—	—	—	—
PAOMY-Mgm-B-cip (16, 16, 16, 16, 32)	<i>nfxB</i>	G538T	G538T	G538T	—	—
	<i>gyrA</i>	G259T	G259T	G259T	G259T	G259T
	<i>gyrB</i>	—	—	—	—	—
	<i>parC</i>	—	—	—	—	—
PAOMY-Mgm-C-cip (16, 16, 16, 16, 32)	<i>nfxB</i>	—	—	—	—	C495A
	<i>gyrA</i>	G259T	G259T	G259T	G259T	G259T
	<i>gyrB</i>	—	—	—	—	—
	<i>parC</i>	—	—	—	—	—

^a ND, not determined; —, no change; del, deletion; pos, position; ins, insertion.

resistant subpopulations (++) growing up to 4 μg/ml and 0.5 μg/ml, respectively, were observed after 240 generations of growth in the presence of 1/4 the MIC of ciprofloxacin, but such subpopulations were not observed in bacterial cultures that evolved in the absence of the quinolone.

The efficacy of tobramycin seemed not to be affected by the decrease in the sensitivity to ciprofloxacin and beta-lactams.

DISCUSSION

The preexistence of antibiotic-resistant mutants in PAOΔmutS hypermutable bacterial populations and the rapid selection of these under monotherapy treatment with ciprofloxacin at high

antibiotic concentrations (0.5 and 1 μg/ml) have been reported previously (20). The resistance mechanisms responsible for the resistance phenotype involved mutations in the quinolone resistance-determining regions (QRDR) *gyrA* and *gyrB* and upregulation of the efflux pumps MexAB-OprM and MexCD-OprJ (21).

Here, we show that exposure at a sub-MIC of ciprofloxacin (0.05 μg/ml, representing 1/4 the MIC) also rapidly selects for preexisting mutants with high-level resistance from *P. aeruginosa* populations. The faster occurrence of ciprofloxacin-resistant mutants in the mutator populations compared to PAO1 is not surprising, as the rate of adaptation depends on both the mutation rate and the population size (22) and only a bottleneck of 10⁸ cells

TABLE 2 Doubling times of PAO1, PAOΔmutS, PAOMY-Mgm, and the ciprofloxacin-resistant mutants in the presence and absence of 0.05 μg/ml ciprofloxacin

Isolate	Doubling time, min (mean ± SD)	
	Without ciprofloxacin	With 0.05 μg/ml ciprofloxacin
Starting strains		
PAO1	24.5 ± 1.9	32 ± 1.2
PAOΔmutS	26.2 ± 0.6	31.4 ± 0.5
PAOMY-Mgm	25.8 ± 0.6	32.3 ± 0
Ciprofloxacin-resistant mutants		
Day 10		
PAO1-A-cip (GyrA T83I)	27.15 ± 0.5	26 ± 0.2
PAO1-B-cip (GyrA D87Y)	26.9 ± 0.07	28 ± 1.9
PAO1-C-cip (GyrB S466Y)	27.2 ± 0.2	27.8 ± 1.2
Day 3		
PAOΔmutS-A-cip (GyrA T83I)	28.9 ± 0	27.8 ± 2.5
PAOΔmutS-B-cip (GyrA T83I)	29.7 ± 1.1	28.2 ± 0.4
PAOΔmutS-C-cip (GyrA T83I)	29.9 ± 1.4	28.6 ± 0.4
PAOMY-Mgm-A-cip (GyrB S466Y)	29.7 ± 1.4	25.7 ± 0.3
PAOMY-Mgm-B-cip (GyrA D87Y)	29.5 ± 0	27.8 ± 0.7
PAOMY-Mgm-C-cip (GyrA D87Y)	28.3 ± 0.2	27.8 ± 0.8

was tested in our study. The high mutation rates that initially confer a benefit because of a faster generation of adaptive mutations might present drawbacks during evolution, probably because the bacteria can accumulate deleterious mutations at a higher rate. The occurrence of trade-offs with disadvantages in secondary environments during the evolution of *Salmonella* in an animal model has previously been reported (22).

In accordance with these results, colonies unable to grow on glucose minimal medium (indicating possible auxotrophs) were found in the mutator populations during the 940 generations in rich LB medium. This indicates that in our long-term evolution study, accumulation of mutations caused a growth disadvantage of the evolved populations in secondary environments. This might explain the lack of transmissibility of mutator strains among CF patients (16) and the reduced potential for colonization of new environments (23, 24). Although the rich LB medium used in our study has a composition different from that of the CF sputum, it is worth mentioning that auxotrophy is a common *P. aeruginosa* phenotype selected during growth in the amino acid-rich environment in the CF sputum (25).

Generally, it is assumed that enrichment of resistant mutants is a consequence of using antibiotic concentrations placed within the mutant selection windows, i.e., the range between the concentration which blocks growth of the majority of susceptible pathogens (MIC) and the concentration that blocks growth of first-step resistant mutants (mutation prevention concentration) (13). However, our results imply that the biologically relevant sub-MIC selective window is much wider and has to include antibiotic concentrations below the MIC of the susceptible population. These concentrations have been shown to be several hundredfold lower than the MIC in *E. coli* (13).

At selection above the MIC, the main driving force of the selection is antibiotic resistance, while the fitness cost of the mutation is less critical. Even mutations with a very high cost will be selected, since competitors in the form of susceptible bacteria will

be eliminated. At sub-MIC levels, however, the situation is different, since the susceptible bacteria will not die; they will only grow slower. Because of this, resistance mutations conferring a high fitness cost will not be enriched; only mutations where the fitness cost is lower than the growth reduction caused by the antibiotic in the susceptible bacteria will be competitive. This suggests that a new spectrum of low-cost or no-cost resistance mutations may be enriched under such conditions (13).

However, at concentrations that only slightly reduce and do not inhibit the bacterial growth, we found the same spectrum of mutations in *gyrA* and *gyrB* as observed under treatment with inhibitory concentrations (21, 26). Combined with the findings of no growth disadvantage of these mutants, these data suggest that the bacterial fitness is not affected by these mutations or that accumulation of compensatory mutations at other loci occurs fast. This is in accordance with previous studies showing that only 3 out of 8 *P. aeruginosa gyrA* mutants were affected in their growth rate (26). Identification of possible loci for compensatory mutations has recently been investigated by Wong et al. (6) by whole-genome sequencing. In that study, the authors found that a possible locus of compensatory mutations could be *nusA*, encoding an elongation factor, a putative kinase, and *ate1*, which encodes an arginyl-tRNA protein.

Enrichment of mutants with a high resistance level has been observed for *Salmonella* and *E. coli* exposed to low-antibiotic concentrations as well (13), probably due to their low (or lack of) fitness cost. Interestingly, the same type of mutations has been identified both *in vitro* and *in vivo* in a large number of ciprofloxacin-resistant clinical *P. aeruginosa* isolates, including strains from patients with cystic fibrosis (21, 27, 28).

Sequencing of the efflux pump regulator gene *nfxB* in ciprofloxacin-resistant isolates selected during the evolution study identified various mutations, especially in the mutator population (Table 1; see Table S1 in the supplemental material). However, no *nfxB* mutants were fixed during the long-term evolution experiment, probably due to the small advantage conferred by these mutations compared to the mutations in *gyrA* and *gyrB*. Mutations in *nfxB*, though, have been frequently found in clinical CF isolates (27), suggesting that these mutants are selected under *in vivo* conditions. The method used to isolate ciprofloxacin-resistant mutants, namely, isolation of resistant colonies in the inhibition zone of ciprofloxacin Etests, which enriches for ciprofloxacin-resistant mutants with high MIC levels, could have created a selection bias for *gyrA* and *gyrB* mutations. A population analysis of the whole bacterial population would have identified bacterial subpopulations with low MIC levels due to upregulation of efflux pumps as previously described (5, 17).

The MICs of resistant mutants selected from the PAOΔmutS mutator population (MIC = 128 μg/ml) was higher than that of the resistant mutants selected in PAO1 (MIC = 32 μg/ml). This is probably due to accumulation of mutations in different genes, such as seen with the *gyrA parC* double mutant that was selected late (after 940 generations) during the evolution experiment in the A and C lineages of PAOΔmutS. The late occurrence of the *parC* mutation confirms previous reports showing that the *parC* mutations are second-step mutations occurring in strains already having alterations in *gyrA* (29). It is noteworthy that strains with two alterations (Thr83Ile in GyrA plus Ser87Leu in ParC) were most frequently identified in clinical isolates (48 isolates) (29).

These findings suggest that mutators have an adaptive advan-

tage also at sub-MIC levels of antibiotics and that the mutant selection window is wider than for wild-type strains, not only toward high (30) but also toward low antibiotic concentrations. A clear correlation between the type of mutations identified and the MIC was, however, difficult to assess, and this is in accordance with previous reports where the same mutations were identified in isolates with a wide range of MICs, probably due to the simultaneous presence of mutations in unidentified loci (21).

Worrisomely, cross-resistance to beta-lactams was observed in bacterial populations treated with ciprofloxacin at sub-MIC levels. This concentration of ciprofloxacin causes formation of reactive oxygen species as shown by measurement of HPF fluorescence. These reactive oxygen species are potentially mutagenic and can cause increased mutability, as previously suggested for strains under ciprofloxacin treatment (12). However, we did not observe an increase in the mutation frequency in the studied bacterial populations, which would especially be expected in the PAOMY-Mgm mutant. This mutant is unable to repair the oxidative DNA lesions that might occur during ciprofloxacin-related ROS exposure.

In conclusion, exposure of *P. aeruginosa* to sub-MICs of ciprofloxacin rapidly selects for mutants with high-level ciprofloxacin resistance and for subpopulations with an increased MIC to unrelated antibiotics. This raises the possibility of long-term persistence of resistant bacteria and spread of antibiotic resistance by exposure of commensal bacterial flora to low antibiotic concentrations.

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