

Fluoroquinolone-Resistant Mutants of *Burkholderia cepacia*[∇]

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Fluoroquinolone-resistant *Burkholderia cepacia* mutants were selected on ciprofloxacin. The rate of mutation in *gyrA* was estimated to be 9.6×10^{-11} mutations per division. Mutations in *gyrA* conferred 12- to 64-fold increases in MIC, and an additional *parC* mutation conferred a large increase in MIC (>256-fold). Growth rate, biofilm formation, and survival in water and during drying were not impaired in strains containing single *gyrA* mutations. Double mutants were impaired only in growth rate (0.85, relative to the susceptible parent).

Exposure to fluoroquinolones increases mutation rates (9, 12, 20, 26) to various degrees (23). The main mechanism of resistance in gram-negative bacteria develops via stepwise accumulation of mutations in the quinolone resistance-determining region (QRDR) of topoisomerase genes (4, 7, 8, 13).

Opportunistic pathogens of the *Burkholderia cepacia* complex (BCC) consist of genomovars that are important in cystic fibrosis patients (14, 17). Genomovars are species which are phylogenetically distinguishable but phenotypically indistinguishable from each other. Here, BCC refers to the complex, while *B. cepacia* refers to genomovar I. BCC bacteria can survive in respiratory droplets on surfaces (6) and are resistant to many antibacterial agents.

Resistance to drying allows maintenance on environmental surfaces (24) and transmission between hosts. Transmission between colonized patients has been documented (18).

The objective of this work was to investigate the effects of fluoroquinolone resistance mutations on growth rate, biofilm formation, and environmental survival.

B. cepacia 10661 (National Collection of Type Cultures, HPA, London, United Kingdom) and mutants derived from this strain were used. The MICs of parent and mutant strains were determined by the ciprofloxacin Etest (AB Biodisk, Solna, Sweden).

The ciprofloxacin MIC of *B. cepacia* 10661 was 1 $\mu\text{g/ml}$. Putative resistant mutants were selected at 2 \times , 4 \times , and 6 \times MIC in three separate experiments. Estimation of the mutation rate was performed using ciprofloxacin at 6 \times MIC. Characterized first-step mutants were used to obtain second-step mutants by selecting first-step mutations on media containing twice the MIC of the first-step mutants. The numbers of viable cells, from three aliquots (approximately 10%), were determined using the method of Miles and Misra in order to determine total cell numbers (3, 11). The plates were incubated at 37°C for 18 h, and the proportion of cultures with mutant colonies were recorded. The mutation rate (μ) was determined using the p_0 method (10, 19, 21).

Approximately 10^2 exponentially growing cells were independently inoculated into 28 tubes, each containing 3 ml of Mueller-Hinton broth (Oxoid, Basingstoke, United Kingdom), and incubated at 37°C for 22 h on an orbital shaker (250 rpm; Barloworld Scientific, Rochester, NY). The cells were harvested by centrifugation (2,000 \times g, 10 min), the supernatant was removed with a pipette, and the pellet was resuspended in 400 μl of Mueller-Hinton broth and then plated onto Mueller-Hinton agar (Oxoid, Basingstoke, United Kingdom) containing ciprofloxacin.

The mutants were characterized by sequencing the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* by using the primers listed in Table 1. Standard PCR conditions were employed. Sequencing was performed by the dideoxy method as previously described (15).

No mutations (0/45) were found in the QRDRs of the topoisomerase genes of *B. cepacia* selected at 2 \times MIC. The MIC of these nontopoisomerase mutants was 4 to 5 $\mu\text{g/ml}$. At 4 \times MIC, an Asp87Asn mutation, conferring a 16-fold increase in MIC, was found in colonies from one plate (2/55). All other mutants (53/55) selected at this concentration contained no mutations in the QRDRs (MICs between 4 and 5 $\mu\text{g/ml}$). All first-step mutants selected at 6 \times MIC (50/50) contained a Thr83Ile mutation in *gyrA* and had an MIC of 64 $\mu\text{g/ml}$. Mutations, MIC, and selection step information are shown in Table 2. Mutation rates for second-step mutations were higher than those for the first-step mutations.

To detect efflux activity, the ciprofloxacin MIC of the fluoroquinolone-resistant mutants was determined in the absence and presence of reserpine (25 $\mu\text{g/ml}$) in Mueller-Hinton agar (2). The MICs of all mutants that did not contain *gyrA* mutations, selected at either 2 \times MIC (45 mutants tested) or 4 \times MIC (55 mutants tested), decreased fivefold in the presence of reserpine to the level of the wild type. The MICs of mutants containing topoisomerase mutations did not decrease.

The quantification of biofilm growth was achieved by the spectrophotometric measurement of crystal violet binding by following a previously published method (5). Mutation in *gyrA* and *parC* did not affect biofilm formation in *B. cepacia*. All fitness assays were carried out with one mutation of each type (data not shown).

We modified the method of Youmans and Youmans (25) to determine time to positivity as an indicator of the growth rate.

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TABLE 1. Primers used to amplify the QRDRs of the topoisomerase genes of *B. cepacia*

Gene	Primer positions ^a	Sequence (5'-3')	Amplicon size (bp)
<i>gyrA</i>	62-81	5' ATCTCGATTACGCGATGAGC	449
	493-511	5' GCCGTTGATCAGCAGGTT	
<i>gyrB</i>	1127-1146	5' GAGGAAGTTGTGGCGAAGG	400
	1502-1520	5' AGTCTTCCTTGCCGATGC	
<i>parC</i>	98-118	5' ATTGGTTCAGGGTCGTGAAGA	229
	295-315	5' GTAGCGCAGCGAGAAATCTT	
<i>parE</i>	1178-1198	5' CAGGGCAAGGTAGTCGAAAA	380
	1557-1577	5' GTGAGCAGCAAGGTCTGGAT	

^a *B. cenocepacia* numbering.

The Bactec 9240 continuous blood culture system with standard aerobic medium (Plus Aerobic/F) was used. Aliquots of 100 μ l of the diluted exponential culture (1/10 and 1/1,000) were removed using a 0.5-ml syringe and a needle and were aseptically inoculated into duplicate culture vials. The length of time to detection (time to positivity) was measured for all strains. Gram staining and a purity plate assay were performed to confirm the absence of contaminants.

The growth rate constant, k , was determined using equation 1 (where A is the largest inoculum employed, B is the smallest inoculum, and t is the difference in time to positivity in hours). The generation time (G) was determined using equation 2.

$$k = \frac{\log A - \log B}{t} \quad (1)$$

$$G = \frac{\log 2}{k} \quad (2)$$

This experiment was repeated in triplicate. The growth rates of the double mutants relative to those of the parent were 0.88 and 0.83 for mutant strains F3 and F4, respectively, as shown in Table 2.

Competition assays were used to measure the fitness of the fluoroquinolone mutants compared to that of the susceptible parent by the use of a modified version of our previously published method (3, 11). The optical densities of the wild-type and mutant isolates were adjusted to the same value (1.0 optical density unit). Then, 250 μ l of each culture was inoculated

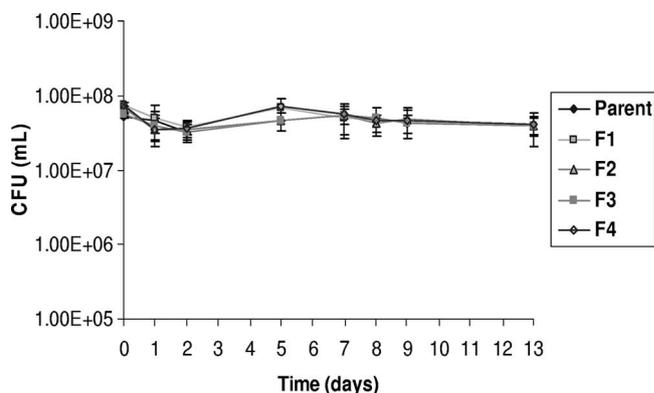


FIG. 1. Effect of topoisomerase mutations on the survival of *B. cepacia* in water. Survival of *B. cepacia* in water was not affected by mutation in gyrase subunit A or topoisomerase IV. Error bars indicate the standard errors of the means. Differences in survival are not significant.

into 15 ml of LB broth in the absence of antibiotics. This mixed culture was incubated for 10 h (200 rpm). The relative fitness of each strain was calculated from the ratio of the number of generations grown by the resistant strains to the number grown by the susceptible strains. Five independent pairwise cultures were performed for each mutant. The relative growth rates of mutant strains F3 and F4 were 0.80 and 0.78, respectively. The differences in relative growth rates of the strains with the single *gyrA* mutations found during paired competition assays were not significant, as determined by Student's t test. However, these assays cannot measure differences of >1%.

Survival in water and survival during drying were assessed using the method employed by Sánchez et al. (22). No significant differences in environmental survival were found between the mutants and the susceptible parent, as shown in Fig. 1 and Fig. 2.

Selection at lower concentrations of fluoroquinolone resulted in mutants in which resistance was apparently due to an altered expression of an efflux pump. Similarly, Zhou et al. demonstrated that low concentrations of fluoroquinolone selected nongyrase mutants of *Mycobacterium smegmatis* (27).

At higher selection concentrations of ciprofloxacin (4 \times and 6 \times MIC), mutations in the topoisomerase genes were found. Lower-level resistance (12- to 64-fold) was caused by single mutations in *gyrA*. Higher-level resistance (MIC of >256 μ g/

TABLE 2. Characteristics of fluoroquinolone-resistant *B. cepacia* mutants selected in vitro^a

Strain	Mutation rate (mutation/division)	MIC (μ g/ μ l)	Selection step	Sequence found in QRDRs of:				Generation time [min (95% confidence interval)]	P value	Relative fitness (\pm SEM) ^b	P value
				<i>gyrA</i>	<i>gyrB</i>	<i>parC</i>	<i>parE</i>				
WT		1		WT	WT	WT	WT	38.0 (37.06-38.94)			
F1 mutant	9.6×10^{-11}	12	1st	Asp87Asn	WT	WT	WT	37.0 (36.77-37.23)	0.331	1.01 ± 0.01	0.831
F2 mutant	9.6×10^{-11}	64	1st	Thr83Ile	WT	WT	WT	37.1 (36.9-37.3)	0.377	1.01 ± 0.152	0.868
F3 mutant	1.1×10^{-10}	>256	2nd	Asp87Asn	WT	Ser80Leu	WT	43.0 (41.85-44.15)	0.004	0.80 ± 0.12	0.003
F4 mutant	6.8×10^{-10}	>256	2nd	Thr83Ile	WT	Ser80Leu	WT	45.7 (44.2-47.2)	0.001	0.78 ± 0.18	0.002

^a Strain F1 was isolated on 2 μ g/ml ciprofloxacin (2 \times MIC); F2 was isolated on 6 μ g/ml (6 \times MIC), F3 was isolated on 24 μ g/ml ciprofloxacin by the use of F1 as the starting point; F4 was isolated on 128 μ g/ml ciprofloxacin by the use of F2 as the starting point. The statistical significance of generation time differences is shown by a P value. WT, wild type.

^b Competition assays were used to measure the fitness of the fluoroquinolone mutants relative to that of the susceptible parent.

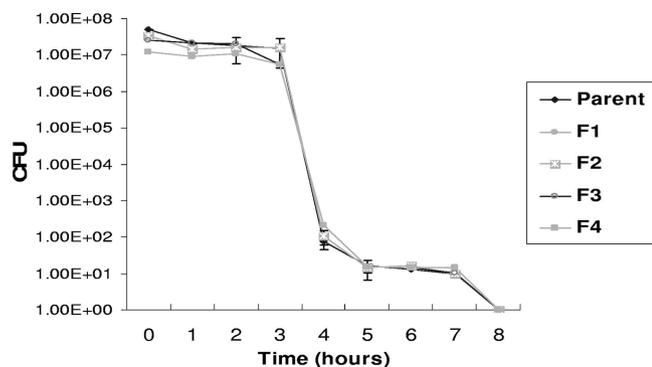


FIG. 2. Effect of topoisomerase mutations on the survival of *B. cepacia* on dry surfaces. Survival of *B. cepacia* in water was not affected by mutation in gyrase subunit A or topoisomerase IV. Error bars indicate the standard errors of the means. Error bars are not shown if obscured by the symbol. Differences in survival are not significant.

ml) required mutations in both *gyrA* and *parC*. The same second-step mutation occurred irrespective of the starting point.

Single-step fluoroquinolone resistance in *gyrA* occurs at low or no cost to *B. cepacia*, and this has been observed for other bacteria (11, 1, 16). These mutants may, therefore, remain in the bacterial population in the absence of an antibiotic selective pressure.

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