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Development of Multiple-Antibiotic-Resistant (Mar) Mutants of *Pseudomonas aeruginosa* after Serial Exposure to Fluoroquinolones

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Laboratory-derived fluoroquinolone-resistant mutants were created by serially passaging wild-type *Pseudomonas aeruginosa* on fluoroquinolone-containing agar to obtain high-level fluoroquinolone resistance (e.g., ciprofloxacin MIC of 1,024 μg/ml). With increases of 4- to 32-fold in MICs of fluoroquinolones, these organisms demonstrated (relative to wild-type) normal morphology, resistance to fluoroquinolones only, no change in fluoroquinolone uptake, and no change in lipopolysaccharide profiles or outer membrane protein profiles. Complementation with wild-type *Escherichia coli gyrA* restored fluoroquinolone susceptibility, suggesting that these were *gyrA* mutants. After 4- to 32-fold increases in fluoroquinolone MICs (with continued passage on fluoroquinolone-containing agar) isolates demonstrated altered morphology, a multiple-antibiotic-resistant (Mar) phenotype (including cross-resistance to beta-lactams, chloramphenicol, and tetracycline), reduced fluoroquinolone uptake and altered outer membrane proteins (reductions in the 25- and 38-kDa bands as well as several bands in the 43- to 66-kDa region). Complementation with wild-type *E. coli gyrA* partially reduced the level of fluoroquinolone resistance by approximately 8- to 32-fold, suggesting that these mutants displayed both *gyrA* and non-*gyrA* mutations.

Fluoroquinolones such as ciprofloxacin, norfloxacin, and ofloxacin have potent broad-spectrum antibiotic activities against various gram-positive and gram-negative organisms, including *Pseudomonas aeruginosa* (21, 23). Fluoroquinolones are known to bind to a DNA gyrase-DNA complex as the intracellular target of these drugs (41, 42). In *Escherichia coli*, DNA gyrase consists of two A and two B subunits (products of the *gyrA* and *gyrB* genes, respectively) and is responsible for catalyzing topological changes in DNA, which serves important functions in DNA replication, transcription, recombination, and repair (6, 21). Inhibition of bacterial DNA gyrase by fluoroquinolones leads to inhibition of DNA synthesis which ultimately leads to cell death (11). The precise explanation of the molecular interactions leading to inhibition of DNA gyrase by fluoroquinolones and to cell death is unknown (43).

Fluoroquinolone resistance in *P. aeruginosa* as a result of mutation has been associated with modification of DNA gyrase and/or alteration in outer membrane permeability (2–4, 7, 10, 12, 14, 20, 24, 27, 29–31, 38, 45, 46). It has been indirectly demonstrated that mutations conferring fluoroquinolone resistance in *P. aeruginosa* may be due to alterations in DNA gyrase, because of the reduced sensitivity to fluoroquinolone inhibition of DNA supercoiling in fluoroquinolone-resistant isolates (10, 22, 29) and reduced inhibition of DNA synthesis by fluoroquinolone-resistant isolates upon exposure to fluoroquinolones (3, 4, 24). More definitive evidence that fluoroquinolone-resistant mutations in DNA gyrase may be due to alterations in *gyrA* comes from complementation studies in which wild-type *gyrA* from *E. coli* was expressed in fluoroquinolone-resistant *P. aeruginosa* and fluoroquinolone susceptibility was restored (39). The *gyrA* gene of *P. aeruginosa* although recently cloned (25) had not been cloned at the time of this study. Genetic characterization by DNA sequencing of *gyrA* from *E. coli* reveals that mutations leading to fluoroquinolone resistance are clustered within a narrow region between nucleotides 199 (Ala-67) and 318 (Gln-106) (21). It has been suggested that mutants possessing a single nucleotide change (point mutation) produce single changes in amino acid sequence which result in an altered DNA gyrase which either reduces the affinity of the fluoroquinolone for the enzyme or blocks the access of the antibiotic to the DNA-DNA gyrase complex (21, 39).

In addition to DNA gyrase changes, fluoroquinolone resistance in *P. aeruginosa* can occur as a result of alterations in the outer membrane (2, 3, 12, 14, 20, 27, 31, 38, 45, 46). Resistance selection with fluoroquinolones results in many instances in changes in outer membrane proteins and is manifested as not only resistance to fluoroquinolones but multiple antibiotic resistance (Mar) including beta-lactams, tetracyclines, and chloramphenicol (2, 3, 12, 27, 37, 38). The role of these membrane changes in resistance and whether the proteins involved are porins, however, are uncertain. In addition, the presence or absence of a relationship between changes in the outer membrane leading to a multiple antibiotic resistance phenotype and interaction with a putative *mar* (multiple-antibiotic-resistant) operon in *P. aeruginosa* is presently undetermined (5).

In order to assess whether serial exposure of *P. aeruginosa* to fluoroquinolones could result in a Mar phenotype and to assess the properties of these phenotypes, we created laboratory-derived fluoroquinolone-resistant mutants by serially passaging wild-type *P. aeruginosa* on agar containing increasing concentrations of fluoroquinolones.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Source (reference)
E. coli S17-1[b]	Pro$^-$ Res$^-$ Mod$^+$ Tp^r Sm^r	Robillard (39)
P. aeruginosa		
3854	FQ susceptible	Clinical isolate
3854r	FQ resistant	This laboratory
4047	FQ susceptible	Clinical isolate
4047r	FQ resistant	This laboratory
4048	FQ susceptible	Clinical isolate
4048r	FQ resistant	This laboratory
PAO2	*ser-3*	Holloway collection[c]
PAO4701	*cfxA2 ser-3*	Robillard (39)
Plasmids		
pLA2917	Cloning vector Km^r Tc^r	Robillard (39)
pNJR3-2	Gyrase A clone (pLA2917)	Robillard (39)

[a] Tp, trimethoprim; Sm, streptomycin; Km, kanamycin; Tc, tetracycline; FQ, fluoroquinolone.

[b] S17-1 is a mobilizing *E. coli* strain which carries the transfer genes of the broad-host-range incompatibility group P plasmid RP4 integrated in its chromosome. This strain can transfer any plasmid containing a P-type Mob site to any gram-negative bacterium.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used or constructed in this study are listed in Table 1 (48). Laboratory-derived fluoroquinolone mutants were created by serially passaging wild-type fluoroquinolone-sensitive *P. aeruginosa* (strains 3854, 4047, and 4048) on fluoroquinolone-containing brain heart infusion (BHI) agar containing increasing concentrations of fluoroquinolone. Specifically, BHI agar plates with ciprofloxacin concentrations starting at one-half the MIC, and doubling thereafter, were used for development of fluoroquinolone-resistant mutants. Laboratory-derived fluoroquinolone-resistant mutants included 3854r, 4047r, and 4048r (all demonstrated the target ciprofloxacin MIC of 1,024 μg/ml).

Isolates 3854 and 3854r, 4047 and 4047r, and 4048 and 4048r were verified as isogenic strains by Southern hybridization with an epidemiological DNA probe (PAK 1.2-kb *Hin*dIII pilin DNA fragment) (method of Ogle et al. [36] with modification).

Antibiotics and reagents. The following antibiotics were obtained from the sources indicated in parentheses: ciprofloxacin (Miles Laboratory, Rexdale, Ontario, Canada); cefotaxime (Hoechst-Roussel Canada Inc., Montreal, Quebec, Canada); cefoxitin, norfloxacin, and imipenem (Merck Sharp and Dohme, Rahway, N.J.); chloramphenicol (Parke-Davis, Ann Arbor, Mich.); gentamicin (Schering, Pointe-Claire, Quebec, Canada); piperacillin (Cyanamid Canada, Baie d'Urfe, Quebec, Canada); tobramycin (Eli Lilly and Co. Canada, Scarborough, Ontario, Canada). Pefloxacin and [^{14}C]pefloxacin (23.6 mCi/mmol) were provided as a generous gift from R. Phillips, Rhone-Poulenc Pharma, Montreal, Quebec, Canada. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was obtained from Sigma Chemical Co., St. Louis, Mo. For fluoroquinolone uptake studies, CCCP at a concentration of 50 μM was added 10 min after addition of the radiolabelled antibiotic (9). The concentration of CCCP was determined not to be bactericidal over the course of the assay.

Media and susceptibility testing. ML broth has been previously described (33). For ML agar plates, 1.5% agar (Difco Laboratories, Detroit, Mich.) was added. Determinations of MIC were made by the macrodilution broth method using doubling dilutions as described by the National Committee for Clinical Laboratory Standards (34). The MIC was defined as the lowest concentration of antibiotic that inhibited the development of visible growth after 16 to 20 h at 37°C. All MIC determinations were performed in duplicate on separate days. Antibiotics (when added for plasmid selection) at the following concentrations were used: tetracycline at 10 μg/ml for *E. coli* and tetracycline at 200 μg/ml for *P. aeruginosa*. During MIC determinations, tetracycline at 200 μg/ml was used for plasmid maintenance.

Electron microscopy. Bacterial cells were stained with 2.5 mmol of phosphotungstic acid adjusted to pH 7.0 with sodium hydroxide or ammonium molybdate adjusted to pH 6.0 with sodium hydroxide and viewed on a Phillips model 201 electron microscope.

Fluoroquinolone uptake. Fifty milliliters of Iso-Sensitest broth was inoculated with an overnight culture of *P. aeruginosa* and allowed to grow at 37°C for several hours. The culture was centrifuged, and the pellet was adjusted to approximately 10^8 CFU/ml. Uptake was initiated by the addition of [^{14}C]pefloxacin at a final concentration of 16 μg/ml (9). Aliquots (0.5 ml) were removed at timed intervals, diluted 1:40 in sterile phosphate-buffered saline (PBS) at 7°C, and filtered through nylon membrane filters (0.45-μm pore size; 25-mm diameter) with a vacuum filtration manifold. Prior to filtration, the filters were soaked in sterile PBS. The filters were washed with 20 ml of sterile PBS, dried for 1 h at 60°C, and placed in Cytoscint scintillation cocktail (ICN, Costa Mesa, Calif.) for counting in an LKB Rackbeta 1217 counter. The level of cell-associated radioactivity was determined after correction for nonspecific binding of the radiolabel to filters in the absence of bacterial cells. The level of total cell protein was determined by the method of Lowry et al. (28).

LPS preparation. Lipopolysaccharide (LPS) was isolated by the method of Darveau and Hancock (8). Preparations were stored at −70°C. Samples were electrophoresed in 15% discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels by the method of Laemmli and Favre (26). Gels were silver stained by the method of Morrisey (32).

Opr preparation. Outer membrane proteins (Opr) of *P. aeruginosa* strains were isolated by the method of Hancock and Nikaido (18). Preparations were stored at −70°C. The protein concentration was determined by the method of Lowry et al. (28).

Outer membrane proteins (50 μg of protein) were suspended in electrophoretic sample buffer (2% SDS, 10% glycerol, 0.5 M Tris-hydrochloride [pH 6.8], 0.002% bromophenol blue with 5% 2-mercaptoethanol). The samples were heated to 100°C for 4 min and were electrophoresed in 15% discontinuous SDS-polyacrylamide gels by the method of Laemmli and Favre (26). The gels were stained with 0.1% Coomassie brilliant blue.

Electrophoretic transfer and immunologic detection of OprF. After SDS-polyacrylamide gel electrophoresis, outer membrane proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.) by using a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories, Mississauga, Ontario, Canada). After blotting, the nitrocellulose membrane was soaked for 1 h at room temperature in PBS with 1% skim milk to saturate additional protein binding sites. The primary monoclonal antibody (MA 4-10 [G10]) was allowed to react for 2 h at 37°C. Peroxidase-conjugated goat anti-mouse secondary antibodies (Bio-Rad Laboratories, Mississauga, Ontario, Canada) were added, and the color reaction was obtained with Bio-Rad horseradish peroxidase color development reagent according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

Analysis of gyrase expression in *P. aeruginosa*. *E. coli* S17-1 containing either pLA2917 or pNJR3-2 was introduced into *P. aeruginosa* by conjugation by the method of Robillard (39). For matings, *E. coli* S17-1 donor strains containing pLA2917 or pNJR3-2 and *P. aeruginosa* recipient strains were grown in ML broth overnight at 32°C with aeration. Donor strains were grown in tetracycline (5 μg/ml). Donor culture (0.5 ml) was added to 0.5 ml of recipient culture in a microcentrifuge tube, and the mixed culture was centrifuged. The pellet (mating mixture) was suspended in 50 μl of 0.15 M NaCl and plated onto ML agar. During 5 h of incubation at 35°C, mating and expression occurred. Three milliliters of 0.15 M NaCl was added to the agar plate, and the mating mixture was harvested and plated onto ML agar containing 200 μg of tetracycline per ml. For laboratory-derived fluoroquinolone-resistant *P. aeruginosa* (3854r, 4047r, and 4048r) recipients, 1,024 μg of tetracycline per ml was added to *Pseudomonas* isolation agar (Difco). *P. aeruginosa* recipient strains that inherited pLA2917 or pNJR3-2 grew in the presence of these concentrations of tetracycline, while plasmid-containing *E. coli* donor strains did not. Transconjugants were purified on selection media. The MICs of several fluoroquinolones were determined for each strain in the presence or absence of each plasmid. The determinations of MICs for strains containing plasmids were performed (in duplicate) in the presence of 200 μg of tetracycline ml.

RESULTS

Susceptibility. The antibiotic susceptibilities of the laboratory-derived fluoroquinolone-resistant isolates are listed in Table 2. Fluoroquinolone-resistant isolates (3854r, 4047r, and 4048r) obtained after passages on fluoroquinolone-containing agar not only exhibited high-level fluoroquinolone resistance but exhibited multiple antibiotic resistance (Mar phenotype) (Table 2). This pleiotropic resistance included various antibiotic classes, including beta-lactams, tetracycline, and chloramphenicol. Twofold increases in MICs occurred with imipenem, while aminoglycoside susceptibilities either did not change or increased twofold (Table 2).

The Mar phenotype did not occur until after the isolates exhibited at least 4- to 32-fold increases in MICs to fluoroquinolones. Thereafter, these isolates demonstrated resistance both to fluoroquinolones and to other antibiotic classes (Table 2).

Figure 1 shows the antibiotic susceptibility of strain 4047 at various levels of fluoroquinolone resistance (ciprofloxacin

TABLE 2. Susceptibilities of laboratory-derived fluoroquinolone-resistant *P. aeruginosa*

Antibiotic	MIC (μg/ml) for laboratory isolates (pre- and postpassaging)					
	3854	3854r	4047	4047r	4048	4948r
Fluoroquinolones						
Ciprofloxacin	0.25	1,024	1.0	1,024	0.125	1,024
Norfloxacin	0.5	2,048	2.0	2,048	0.5	2,048
Pefloxacin	2.0	4,096	4.0	2,048	1.0	4,096
Beta-lactams						
Cefoxitin	64	512	32	256	64	1,024
Cefotaxime	32	256	32	512	32	256
Piperacillin	16	128	16	256	16	128
Imipenem	2.0	4.0	4.0	8.0	2.0	4.0
Aminoglycosides						
Gentamicin	4.0	8.0	4.0	4.0	4.0	8.0
Tobramycin	1.0	2.0	1.0	2.0	2.0	2.0
Other antibiotics						
Chloramphenicol	32	256	32	512	64	512
Tetracycline	32	1,024	64	1,024	32	512

MICs of 1, 4, 16, 64, and 256 μg/ml). When the ciprofloxacin MIC was ≤8 μg/ml, these phenotypes demonstrated unchanged susceptibilities to various beta-lactams (e.g., cefoxitin, cefotaxime, and piperacillin) as well as other antibiotics such as tetracycline and chloramphenicol. Once strain 4047 displayed a ciprofloxacin MIC of 16 μg/ml, it demonstrated a Mar phenotype (Fig. 1).

Stability of fluoroquinolone resistance. Laboratory-derived fluoroquinolone-resistant mutants (3854r, 4047r, and 4048r) were passaged 40 times on antibiotic-free BHI agar. Laboratory-derived fluoroquinolone-resistant isolates demonstrated a reduction in the level of resistance on the first few passages; however, they stabilized and then still retained high-level fluoroquinolone-resistance (MICs of 16 to 256 μg/ml). No strain lost its Mar phenotype upon passaging.

Growth and morphology. All wild-type strains (strains 3854, 4047, and 4048) grew quickly, with doubling times in BHI broth of approximately 30 min. Laboratory-derived fluoroquinolone-resistant mutants (3854r, 4047r, and 4048r) grew more slowly than wild-type isolates, with doubling times ranging from approximately 40 min to 1.5 h.

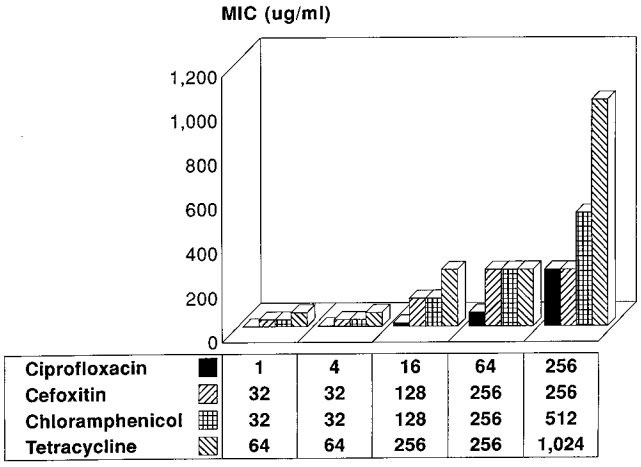

FIG. 1. Antibiotic susceptibility of strain 4047 at various (-fold) levels of fluoroquinolone resistance.

Morphological analysis was performed by both light microscopy and electron microscopy. On Gram strain, all wild-type isolates (strains 3854, 4047, and 4048) demonstrated good strain uptake and typical gram-negative bacillary morphology. Gram stain of laboratory-derived fluoroquinolone-resistant mutants revealed poor staining with loss of rod-like shape and actual rounding of cells. Electron microscopy demonstrated the characteristic gram-negative bacillary appearance of wild-type isolates (strains 3854, 4047, and 4048). Laboratory-derived fluoroquinolone-resistant mutants (Mar phenotype), however, displayed a loss of rod-like shape and had a rounded, coccus-like appearance (Fig. 2). In addition, many cells showed large blebs in the outer membrane (Fig. 2). These gross morphological changes in the laboratory-derived fluoroquinolone-resistant mutants began to appear after these organisms demonstrated at least 4- to 32-fold increases in MICs to fluoroquinolones. For example, isolate 4047 (ciprofloxacin MIC of 1.0 μg/ml) began to show these morphological changes once the ciprofloxacin MIC attained 16 μg/ml.

Uptake of pefloxacin. The fact that the laboratory-derived fluoroquinolone-resistant isolates were not only resistant to fluoroquinolones but also resistant to other groups of antibiotics suggested altered membrane permeability. We therefore studied the uptake of pefloxacin in these isolates. Compared with the pefloxacin uptake of their respective parental strains (strains 3854, 4047, and 4048), laboratory-derived fluoroquinolone-resistant isolates (3854r, 4047r, and 4048r) demonstrated significantly reduced uptake of pefloxacin. Reductions in fluoroquinolone uptake did not occur until after 4- to 32-fold increases in MICs to fluoroquinolones occurred. Figure 3 depicts the uptake of pefloxacin into strain 4047 at various levels of fluoroquinolone resistance. At ciprofloxacin MICs of ≤8 μg/ml, no changes in pefloxacin uptake were noted. However, at ciprofloxacin MICs of ≥16 μg/ml reductions in fluoroquinolone uptake occurred.

Addition of the energy inhibitor CCCP enhanced pefloxacin uptake by 30 to 40% after 30 min for all strains, whether fluoroquinolone-sensitive or -resistant, suggesting that *P. aeruginosa* possesses an active efflux system for fluoroquinolones. In addition, these data suggest that this fluoroquinolone efflux system is intact even in fluoroquinolone-resistant isolates.

LPS. LPS profiles of the parental fluoroquinolone-sensitive (strain 3854, 4047, and 4048) and laboratory-derived fluoroquinolone-resistant (3854r, 4047r, and 4048r) isolates are depicted in Fig. 4. No significant differences between paired isolates were noted.

Outer membrane proteins. Outer membrane protein profiles of fluoroquinolone-sensitive strains (strains 3854, 4047, and 4048) and those of their laboratory-derived fluoroquinolone-resistant paired isolates (3854r, 4047r, and 4048r) were considerably different (Fig. 5). Laboratory-derived fluoroquinolone-resistant isolates demonstrated reduced intensity of bands in the 25- and 38-kDa region. In addition, several bands with molecular masses ranging from 43 to 66 kDa showed reduced intensity in the laboratory-derived fluoroquinolone-resistant isolates. One new band was visualized in strains 3854r and 4048r. The outer membrane protein had an approximate molecular mass of 40 kDa (Fig. 5). It should be noted that, as with the antibiotic susceptibility, morphological analysis, and fluoroquinolone uptake studies, changes in outer membrane proteins did not occur until after the laboratory-derived isolates demonstrated 4- to 32-fold increases in fluoroquinolone MICs. For example, with strain 4047, changes in outer mem-

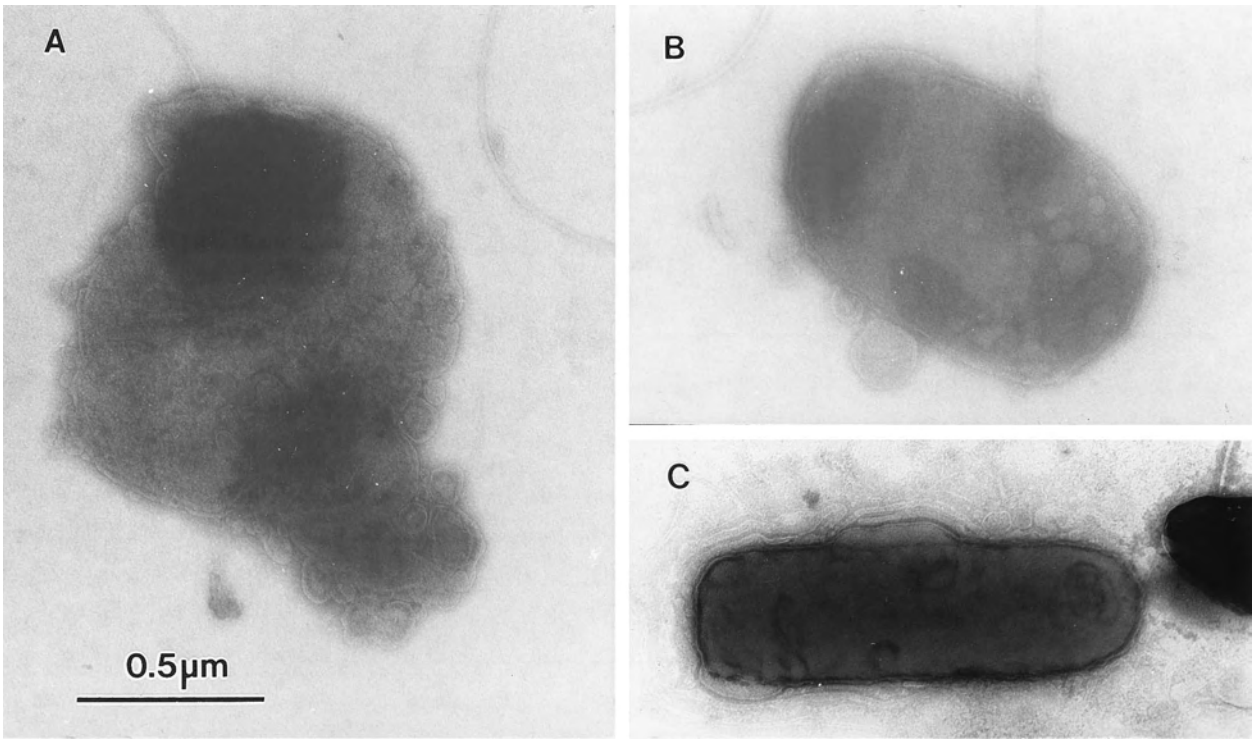

FIG. 2. Electron micrograph showing laboratory-derived fluoroquinolone-resistant *P. aeruginosa* (4047r) (A and B) and a wild-type fluoroquinolone-sensitive *P. aeruginosa* control (strain 4047) (C). All micrographs were taken at equal magnifications (×50,000).

brane proteins did not occur until the MIC of ciprofloxacin became ≥16 μg/ml. Use of an OprF-specific monoclonal antibody (MA 4-10 or G10) either failed to detect OprF (3854r) or demonstrated a reduced signal (4047r and 4048r) in isolates compared with their fluoroquinolone-sensitive isogenic partners (Fig. 6). The new band (molecular mass of ~40 kDa in 3854r and 4048r) did not exhibit a positive signal with OprF-specific monoclonal antibody.

Analysis of *E. coli* gyrase A expression in *P. aeruginosa*. Plasmids pLA2917 and pNJR3-2 were introduced via conjugal mating into the gyrase A^+ strain PAO2 and a known gyrase A mutant (PAO4701), as well as fluoroquinolone-sensitive and -resistant isolates. The fluoroquinolone MICs for these strains are presented in Table 3. As expected, vector pLA2917 had no effect on PAO2, PAO4701, or any other isolate. Also as expected, pNJR3-2 had no effect on PAO2 but did confer fluoroquinolone susceptibility on PAO4701. Plasmid pNJR3-2 reduced the level of fluoroquinolone resistance approximately 8- to 32-fold for the laboratory-derived fluoroquinolone-resistant isolates. This suggested that these isolates carried mutations in *gyrA* as well as other non-*gyrA* mutations. Figure 7 describes the ciprofloxacin susceptibilities of strain 4047 both before and after complementation with *E. coli gyrA*. At ciprofloxacin MICs of ≤8 μg/ml, complementation with *E. coli gyrA* restored ciprofloxacin susceptibility, suggesting *gyrA* mutation. At ciprofloxacin MICs of ≥16 μg/ml, complementation with *E. coli gyrA* restored only partial susceptibility, suggesting both *gyrA* and non-*gyrA* mutations.

DISCUSSION

In this study, we created laboratory-derived fluoroquinolone-resistant mutants of *P. aeruginosa* by serially passaging

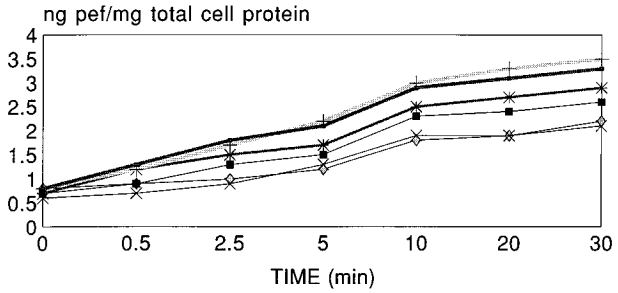

FIG. 3. Uptake of [^{14}C]pefloxacin into strain 4047 at various levels of fluoroquinolone resistance. Values for isolates with ciprofloxacin MICs of 1 (□; wild type), 4 (+), 16 (*), 64 (■), 256 (×), and 1,024 (◇) μg/ml were calculated. pef, pefloxacin.

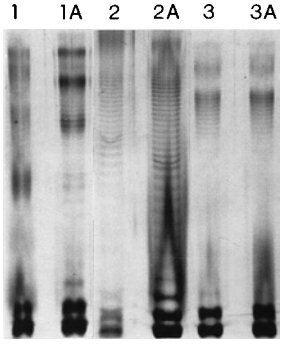

FIG. 4. LPS analysis of fluoroquinolone-sensitive strains (strains 3854, 4047, and 4048 [lanes 1, 2, and 3, respectively]) and laboratory-derived fluoroquinolone-resistant isolates (3854r, 4047r, and 4048r [lanes 1A, 2A, and 3A, respectively]) of *P. aeruginosa*.

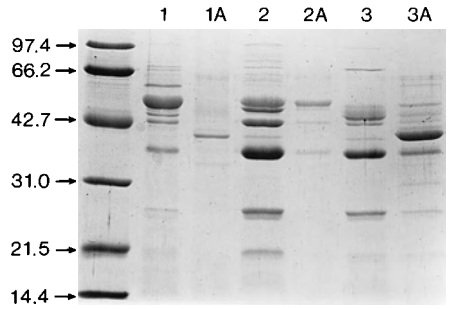

FIG. 5. Outer membrane protein profiles of fluoroquinolone-sensitive strains (strains 3854, 4047, and 4048 [lanes 1, 2, and 3, respectively] and laboratory-derived fluoroquinolone-resistant isolates (3854r, 4047r, and 4048r [lanes 1A, 2A, and 3A, respectively]) of *P. aeruginosa*. The numbers on the left indicate the molecular weights (in thousands) of standard proteins (arrows).

wild-type organisms on agar containing increasing concentrations of fluoroquinolone. We hypothesized that continued passage on fluoroquinolone agar would lead to a Mar phenotype. Our laboratory-derived fluoroquinolone-resistant mutants had both DNA gyrase changes (as evidenced by the partial restriction of fluoroquinolone susceptibility upon complementation with *E. coli gyrA*) and permeability alterations. In passaging our isolates on fluoroquinolone-containing agar, none of these properties (altered morphology and growth, reduced fluoroquinolone uptake, multiple antibiotic resistance, and outer membrane protein changes) occurred until after 4- to 32-fold increases in the MICs of fluoroquinolones occurred. As an example, strain 4047 derivatives appeared to be DNA gyrase mutants when they had ciprofloxacin MICs of $\leq$8 μg/ml, as evidenced by complementation with *E. coli gyrA* (Fig. 7). Whether the DNA gyrase mutations at ciprofloxacin MICs of 2, 4, and 8 μg/ml (wild-type MIC, 1 μg/ml) represent one or more mutations is unclear. With continued serial passage, when strain 4047 derivatives achieved ciprofloxacin MICs of 16 μg/ml, they demonstrated a Mar phenotype with resistance not only to fluoroquinolones but also to chemically unrelated classes of antibiotics, including beta-lactams, chloramphenicol, and tetracycline (Fig. 1). We present evidence here that the mutation(s) conferring higher levels of fluoroquinolone resistance were associated with altered morphology, reduced fluoroquinolone uptake, alterations in outer membrane proteins, and a Mar phenotype.

Mutations affecting quinolone susceptibility include *nalA*, *nfxA*, and *cfxA*, which are alleles of *gyrA* that encode A subunits of DNA gyrase that are less susceptible to quinolones (3, 21).

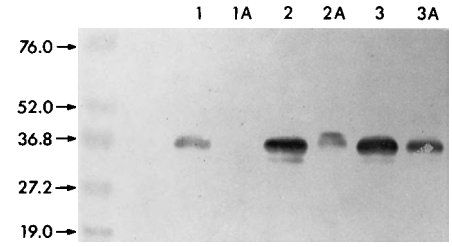

FIG. 6. Western blot (immunoblot) of a replicate of the polyacrylamide gel shown in Fig. 5, immunostained with OprF-specific monoclonal antibody (MA 4-10, G10). Lane 1, strain 3854; lane 1A, isolate 3854r; lane 2, strain 4047; lane 2A, isolate 4047r; lane 3, strain 4048; lane 3A, isolate 4048r. The numbers on the left indicate the molecular weights (in thousands) of prestained standard proteins (arrows).

TABLE 3. Expression of *E. coli gyrA* in *gyrA*$^+$ and *gyrA* *P. aeruginosa* strains

Strain (plasmid)	MIC (μg/ml)		
	Ciprofloxacin	Norfloxacin	Pefloxacin
PAO2 (no plasmid)	0.25	1.0	1.0
PAO2 (pLA2917)	0.25	1.0	1.0
PAO2 (pNJR3-2)	0.25	1.0	1.0
PAO4701 (no plasmid)	2.0	8.0	16.0
PAO4701 (pLA2917)	2.0	8.0	16.0
PAO4701 (pNJR3-2)	0.25	1.0	1.0
3854 (no plasmid)	0.25	0.5	2.0
3854 (pLA2917)	0.25	0.5	2.0
3854 (pNJR3-2)	0.25	0.5	2.0
3854r (no plasmid)	1,024	2,048	4,096
3854r (pLA2917)	1,024	2,048	4,096
3854r (pNJR3-2)	32	64	64
4047 (no plasmid)	1.0	2.0	2.0
4047 (pLA2917)	1.0	1.0	4.0
4047 (pNJR3-2)	1.0	2.0	4.0
4047r (no plasmid)	1,024	1,024	2,048
4047r (pLA2917)	512	2,048	2,048
4047r (pNJR3-2)	64	64	128
4048 (no plasmid)	0.125	0.5	1.0
4048 (pLA2917)	0.125	1.0	2.0
4048 (pNJR3-2)	0.25	1.0	1.0
4048r (no plasmid)	1,024	2,048	4,096
4048r (pLA2917)	512	1,024	4,096
4048r (pNJR3-2)	128	256	512

Other mutations in *P. aeruginosa* affecting quinolone susceptibility but not DNA gyrase have been described (14, 40). *nalB* mutants demonstrate not only resistance to quinolones but also cross-resistance to beta-lactams and chloramphenicol (14, 40). However, it should be noted that *nalB* mutants display various outer membrane protein profiles (e.g., a new 54-kDa outer membrane protein [20], lack of a 31.5-kDa outer membrane

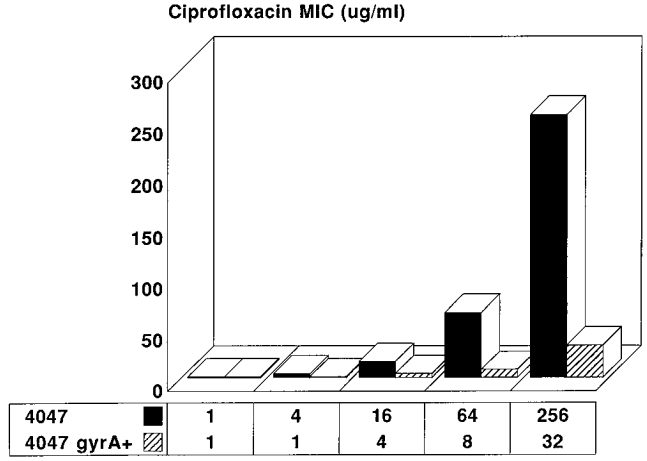

FIG. 7. Ciprofloxacin susceptibilities of strain 4047 (at various (-fold) levels of fluoroquinolone resistance) both before (4047) and after (4047 gyrA$^+$) complementation with *E. coli gyrA*.

protein [7, 24]). The Mar mutants created in this study have similarities to *nalB*.

Piddock et al. (37) have recently described a posttherapy enoxacin-resistant *P. aeruginosa* mutant with biological characteristics very similar to those of our fluoroquinolone-derived Mar mutants. Their posttherapy enoxacin-resistant sputum isolate demonstrated a Mar phenotype with increased resistance to fluoroquinolones and also beta-lactams (cefsulodin, cefotaxime, ceftazidime, carbenicillin, and mezlocillin), chloramphenicol, and tetracycline. Resistance did not occur with aminoglycosides or imipenem. Isolates with this Mar phenotype grew more slowly than the pretherapy isolates. Outer membrane proteins were altered, with OprF totally absent, as confirmed by immunoblots using monoclonal antibody. These investigators suggested that, since the acquisition of fluoroquinolone resistance was associated with the development of a Mar phenotype, these multiple phenotypic changes were somehow genetically linked (37). We believe that a similar scenario occurred with our fluoroquinolone-derived Mar mutants. That is, initial fluoroquinolone exposure led to the development of fluoroquinolone-resistant mutants; however, continued serial fluoroquinolone exposure led to genetically linked changes resulting in a Mar phenotype.

It has been suggested that exposure to fluoroquinolones resulting in not only fluoroquinolone resistance but also multiple antibiotic resistance may occur in one of two ways (37): either (i) the resistant mutants contain an altered regulatory locus that influences several other genes involved in antibiotic resistance or (ii) a single dominant mutation stabilizes other mutations, which upon reversion of this dominant mutation leads to the loss of the effects of the other mutations. An example of the former is *marA* in *E. coli*, where a mutation in the putative regulatory locus affects the expression of other loci in the *marRAB* operon (5).

The marked changes in OprF (Fig. 5 and 6) may be responsible for the gross morphological alterations in these Mar cells. This idea is based on the fact that OprF has important structural functions in the outer membrane (16, 44) and strong noncovalent interactions with both peptidoglycan and LPS (1, 17). Thus, alterations in OprF may cause detachment of the outer membrane from peptidoglycan resulting in protrusion and bleb formation (17). DNA sequencing studies have revealed homology between OprF of *P. aeruginosa* and OmpA (major structural protein) of *E. coli*.

Whether alterations in OprF have led to reduction in fluoroquinolone uptake and multiple antibiotic resistance is unclear. Presently, data suggesting that OprF is a porin that leads to antibiotic resistance (35, 38, 45) and also that OprF is not a porin and is not responsible for antibiotic resistance exist (4, 47). We plan to test our hypothesis regarding the structural role of OprF as well as its role in fluoroquinolone uptake and antibiotic resistance by inserting an expression vector containing the cloned OprF gene into our laboratory-derived fluoroquinolone-resistant isolates and testing for function. In addition, we are presently sequencing OprF from our mutants to assess potential sites of mutation (13). Many other outer membrane proteins in the 43- to 66-kDa range were altered in the laboratory-derived fluoroquinolone-resistant isolates. Whether these proteins are involved with quinolone uptake and the development of multiple antibiotic resistance is unknown (19, 30).

The observation of a multiple-antibiotic-resistant (Mar) phenotype in our laboratory-derived fluoroquinolone-resistant *P. aeruginosa* is important. As previously discussed, the Mar phenotype in clinical isolates has been described before (37). Thus, treatment of patients with *P. aeruginosa* infections with a fluoroquinolone could result in not only fluoroquinolone-resistant organisms but a pathogen resistant to a variety of antibiotics of unrelated chemical classes (Mar phenotype).

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