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A Pleiotropic, Posttherapy, Enoxacin-Resistant Mutant of *Pseudomonas aeruginosa*

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An enoxacin-resistant *Pseudomonas aeruginosa* mutant (G49) isolated during patient therapy was characterized in detail. The G49 mutant was cross resistant to several classes of antibiotics including quinolones, β-lactams, chloramphenicol, and tetracycline, but not imipenem or aminoglycosides. Compared with its paired pretherapy isolate G48, this mutant had several alterations in outer membrane proteins including a complete loss of the major porin protein OprF and a substantially altered lipopolysaccharide profile. Revertants were selected at a frequency of approximately 1% after enrichment for OprF$^+$ cells on low-salt proteose peptone no. 2 medium. Ninety-seven of these OprF$^+$ revertants were as susceptible to carbenicillin and norfloxacin as the pretherapy isolate. One of these revertants was characterized in more detail and shown to be indistinguishable in all properties from the pretherapy isolate. It is proposed that the multiple-antibiotic-resistance (Mar) phenotype of this mutant resulted from a single pleiotropic mutation.

Enoxacin is a difluorinated quinolone with strong activity for gram-negative bacteria (7, 41). Enoxacin is now marketed in 11 countries, including South America, the United Kingdom, and other parts of Europe, for use in the therapy of urinary tract infections and in some countries for respiratory tract infections. As in other bacteria, the primary target for quinolones in *Pseudomonas aeruginosa* is DNA gyrase. *nalA*, *nfxA*, *norA*, and *cipA* are alleles of *gyrA* and encode A subunits that are less susceptible to inhibition by quinolones (14, 16, 33, 34). Other mutations affecting quinolone activity, but not DNA gyrase, in *P. aeruginosa* have also been described. Many laboratory mutants contain alleles of *nalB* and require nalidixic acid MICs of >500 μg/ml and are cross resistant to carbenicillin, ureidopenicillins, chloramphenicol, and novobiocin (33, 34). Resistant strains have arisen during experimental *P. aeruginosa* infections, with several classes of mutants isolated with mutations resembling *nalA* and *nfxC* (10, 22, 23). Quinolone-resistant *P. aeruginosa* isolated from patients may also have the *nalA* or *nalB* phenotype (43) or a *nalB*-like phenotype lacking OprF. Such strains have been isolated from patients with chronic obstructive airway disease (30), burn wound sepsis (17), cystic fibrosis (6), and empyema (20). While several strains with decreased susceptibilities to several agents have been isolated, which suggests a mutation in *nalB*, there are biochemical differences that are commensurate with different mutations encoding multiple resistance. Some quinolone-resistant *P. aeruginosa* strains contain a new 54-kDa outer membrane protein (14, 18) or lack a 31.5-kDa outer membrane protein (9, 17), possibly OprF, or have decreased levels of proteins D2 and H1 or G1 (5, 20, 23). Several workers have also reported changes in lipopolysaccharide (LPS) (6, 18, 34).

An open study to evaluate the efficacy of enoxacin in respiratory tract infections with particular attention to those caused by *P. aeruginosa* was performed (40). During this study posttherapy sputum specimens from 12 patients yielded *P. aeruginosa* strains with decreased susceptibilities to enoxacin and other fluoroquinolones (31). Some strains also showed decreased susceptibilities to chemically unrelated antimicrobial agents. In this study we have characterized the mechanisms of resistance exhibited by an apparently isogenic pair of pre- and posttherapy *P. aeruginosa* isolates.

MATERIALS AND METHODS

Strains. Of 12 pairs of pre- and post-enoxacin therapy *P. aeruginosa* isolates from the sputum samples of patients treated with enoxacin and obtained from W. J. A. Wijnands (Foundation Deventer, Deventer, The Netherlands), 1 pair (the pretherapy isolate G48 and posttherapy isolate G49), described in brief previously (31), were selected for detailed study. This pair of isolates had the same serotype, pyocin type, and phage type (39). These data together with the revertant analysis described in Results suggested that G48 and G49 were isogenic. From strain G49, the revertant G49R2 was isolated after overnight growth of G49 on low-salt Luria broth, which selects against growth of OprF$^-$ strains (42). Strain G49R2 was identified among survivors by screening for the presence of OprF by using colony immunoblotting and anti-OprF monoclonal antibody MA4-4 as described by Mutharia and Hancock (25).

Media, antibiotics, and susceptibility determination. Isosensitest medium (Unipath, Basingstoke, United Kingdom), Luria broth, or proteose peptone no. 2 broth supplemented with 10 or 200 mM sodium chloride was used as the rich medium. Minimal Broth Davis (Difco) supplemented with 0.4% Casamino Acids, 0.2% glucose, and 0.01% thiamine hydrochloride was used throughout as minimal medium. Antimicrobial agents were obtained from their manufacturers or from Sigma Chemical Co. (St. Louis, Mo.). Susceptibility testing was performed at 37°C by a standard agar doubling dilution method with an inoculum of 10^4 and 10^6 CFU per spot or by the microdilution method in Mueller-Hinton broth (36). Very similar results were obtained with both procedures.

Outer membrane proteins and LPS analysis. Outer mem-

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TABLE 1. Antibiotic susceptibility of *P. aeruginosa* isolates and revertant

Strain	Derivation	MIC[a] (μg/ml)											
		ENX	CIP	CFS	CTX	CAZ	IMP	MEZ	CARB	CPO	SM	CML	TET
G48	Pretherapy	0.3	0.08	0.2	0.4	0.4	0.4	4	0.8	0.8	0.8	32	8
G49	Posttherapy	3.0	0.8	0.8	25	1.5	0.4	64	50	6	0.8	128	32
G49R2	Revertant	0.3	0.08	0.2	0.4	0.4	0.4	ND[b]	0.8	0.8	0.8	ND	ND

[a] Abbreviations: ENX, enoxacin; CIP, ciprofloxacin; CFS, cefsulodin; CTX, cefotaxime; CAZ, ceftazidime; IMP, imipenem; MEZ, mezlocillin; CARB, carbenicillin; CPO, cefpirome; SM, streptomycin; CML, chloramphenicol; TET, tetracycline.
[b] ND, not done.

branes were isolated, and all samples were electrophoresed on vertical 10 (29) or 14% (12) polyacrylamide–sodium dodecyl sulfate (SDS) gels. LPS was analyzed as described by Hitchcock and Brown (15) with and without the modifications described by Lesse et al. (19) and stained by the method of Tsai and Frasch (38). The nomenclature and apparent molecular sizes of *P. aeruginosa* outer membrane proteins have been described previously (13).

Accumulation of quinolones. Cells were grown to mid-exponential phase (A_{470} = 0.6 to 0.8), and the accumulation of enoxacin or norfloxacin in the presence and absence of 7.5 mM EDTA or 20 μM carbonyl cyanide *m*-chlorophenylhydrazone was measured by using the fluorometric assay described by Mortimer and Piddock (24). The fluorescence of the supernatant was measured at excitation and emission wavelengths of 342 and 411 nm, respectively, for enoxacin and 281 and 440 nm, respectively, for norfloxacin. By comparison with standard curves of fluorescence versus micrograms of quinolone and of viable count versus milligrams (dry weight) of each isolate, the uptake data were converted and expressed as nanograms of quinolone per milligram (dry weight) of cells.

Measurement of DNA synthesis. The isolates were grown in minimal medium to an A_{470} of 0.5 and assayed as described by Benbrook and Miller (3). By using the linear portion of the inhibition curve, the concentrations of quinolone with and without 7.5 mM EDTA to inhibit DNA synthesis by 50 and 90% were calculated.

β-Lactamase analysis and plasmid profiles. Crude β-lactamase extracts were analyzed by isoelectric focusing as described by Matthew et al. (21). The kinetics of β-lactamase hydrolysis were analyzed from noninduced cells using nitrocefin as a substrate, and the K_m and V_{max} were calculated. All assays were performed at 37°C using an LKB Ultrospec with a heated cell, and the data were analyzed as described by Piddock et al. (29). Inducibility was examined by the zone blunting method (35). Plasmids were extracted and visualized by agarose electrophoresis essentially as described by Bennett et al. (4).

RESULTS

Phenotypic characteristics of strains G48 and G49. The posttherapy isolate G49 had a complex phenotype. It demonstrated a 10-fold decrease in quinolone susceptibility compared with the pretherapy isolate G48, in addition to 4- to 60-fold increases in the MICs of several β-lactams (Table 1). However, these changes in MIC were not completely nonspecific. For example, imipenem, which utilizes a specific porin, OprD (37), and the aminoglycosides streptomycin (Table 1), gentamicin (MIC = 0.25 μg/ml) and amikacin (MIC = 1 μg/ml), which utilize the self-promoted uptake pathway (13), had similar MICs for both G48 and G49.

Treatment of *P. aeruginosa* infections with β-lactams often results in mutants derepressed for chromosomal β-lactamase, resulting in broad cross-resistance to many β-lactams, with the exception of imipenem. However, this was not the case for strains G48 and G49, which contained basal levels of a single β-lactamase with a pI of 8.8, the known isoelectric point of *P. aeruginosa* chromosomal β-lactamase. The K_m for β-lactamase (nitrocefin was the substrate) was 6.2 μM and the V_{max} was 20.4 μmol/min/mg of protein for strain G48, whereas the K_m was 6.2 μM and the V_{max} was 16.9 μmol/min/mg of protein for strain G49. Furthermore, application of the zone blunting method of Sanders and Sanders (35) indicated that the β-lactamase from these two strains had similar inducibility profiles with imipenem. No plasmid-mediated β-lactamases were observed for either strain, and indeed a direct examination failed to demonstrate plasmid acquisition by strain G49.

Outer membrane protein and LPS profiles. The outer membrane protein profiles showed substantial changes in strain G49 compared with strain G48 (Fig. 1). Outer membrane protein OprF was totally missing in G49, as confirmed by Western immunoblots using monoclonal antibody MA4-4. Further confirmation that the minor bands seen at the apparent molecular mass of OprF in Fig. 1, lane 2, were not OprF was the demonstration that unlike OprF (12), they were unmodified by treatment with 2-mercaptoethanol. In addition, the amount of a protein of approximately 70 kDa which was not heat modifiable, and thus probably not OprC (13), was reduced, as was the imipenem-selective porin OprD. The amount of an outer membrane protein with an apparent molecular mass of 48 kDa was increased. The

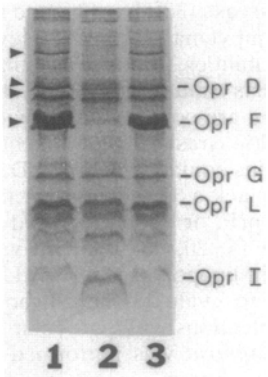

FIG. 1. SDS-polyacrylamide gel electrophoretogram of outer membrane proteins from the pretherapy isolate G48 (lane 1), posttherapy isolate G49 (lane 2), and revertant G49R2 (lane 3). Outer membrane proteins are identified on the right. Arrowheads indicate changes in Opr profiles between lane 2 and lanes 1 and 3.

TABLE 2. Phenotype of pretherapy isolate G48, posttherapy isolate G49, and revertant G49R2

Strain	Presence of OprF in the outer membrane[a]	Doubling time (min) in Luria broth		Enoxacin inhibition of DNA synthesis[b]:			
				With no EDTA		With 7.5 mM EDTA	
		Low salt (10 mM NaCl)	High salt (200 mM NaCl)	IC_{50}	IC_{90}	IC_{50}	IC_{90}
G48	+	31	37	0.41	0.76	0.079	0.15
G49	−	95	45	12.86	26.85	6.23	11.53
G49R2	+	33	34	0.23	0.53	0.079	0.15

[a] As determined by SDS-polyacrylamide gel electrophoresis and Western immunoblotting.
[b] IC_{50} and IC_{90}, concentrations of enoxacin required to inhibit whole-cell DNA synthesis by 50 and 90%, respectively.

amounts of outer membrane proteins OprG, OprH, OprL, and OprE were apparently unchanged.

Strain G49 grew much more slowly than the pretherapy isolate G48 in most media including low-salt Luria broth (Table 2) or proteose peptone no. 2 broth. However, in the presence of high-salt broth, G49 grew almost as rapidly as G48, a characteristic consistent with the identification of G49 as an outer membrane protein OprF-deficient mutant (30). This slow growth characteristic on low-salt medium was utilized to enrich for $OprF^+$ revertants, since no observable revertants arose spontaneously during growth on a high-salt medium. Strain G49 was grown overnight on low-salt Luria or proteose peptone no. 2 broth and subsequently plated for single colonies on Luria or proteose peptone no. 2 agar plates, respectively. These colonies were screened with monoclonal antibody MA4-4 to identify revertants producing OprF. In three separate experiments, the frequencies of the OprF-producing revertants were 1 in 1,000 (Luria broth selection), 1 in 108, and 1 in 130 (proteose peptone broth selection). All revertants grew rapidly and formed large colonies after 18 h, whereas all OprF-deficient strains grew slowly, forming small colonies after 36 h. Ninety-seven $OprF^+$ colonies were picked and tested for susceptibilities to carbenicillin and norfloxacin. All had regained wild-type susceptibility. One revertant, G49R2, was shown to have regained wild-type G48 susceptibilities to all tested antibiotics (Table 1), wild-type growth rates on low-salt medium (Table 2) and normal levels of OprF in the outer membrane (Fig. 1). All of the above-noted outer membrane changes of G49 coreverted in strain G49R2 (Fig. 1). One other $OprF^+$ revertant, G49R1, was isolated inadvertently from survivors of stocks frozen at −70°C in 8% dimethyl sulfoxide. This revertant presumably arose because of the known cold lability of OprF-deficient strains (26). Strain G49R1 exhibited small decreases in the MICs of antibiotics, but not to the levels observed for G48, a 30-fold increase in imipenem resistance, multiple alterations in outer membrane protein pattern compared with both G48 and G49, and a partial reversion of the enoxacin resistance of whole-cell DNA synthesis compared with that of G49. Therefore, it did not appear to be a true genotypic revertant but probably contained a compensatory mutation(s) and thus was not considered further in this study.

Analysis of LPS profiles by the technique of Hitchcock and Brown (15) revealed that G49 had a reduced level of longer-chain species of LPS and an increased concentration of shorter-chain species (Fig. 2A). Application of the method of Lesse et al. (19), to permit subfractionation of rough LPS species comprising the lipid A and rough core oligosaccharides, the majority species in *P. aeruginosa* LPS (2), indicated an alteration in the distribution of species of rough LPS molecules (Fig. 2B). The LPS gel pattern of revertant G49R2 was similar to that of strain G48.

Whole-cell–drug interaction studies. Strain G49 had a reduced susceptibility to enoxacin inhibition of whole-cell DNA synthesis (Table 2). EDTA treatment resulted in a two- to fivefold increase in DNA susceptibility of DNA synthesis to enoxacin inhibition. However, both in the absence and presence of 7.5 mM EDTA, G49 required at least 25-fold more enoxacin to inhibit DNA gyrase in vivo than did either the pretherapy isolate G48 or the revertant G49R2. Experiments were performed to determine whether strain G49 had a deficiency in accumulation of enoxacin and norfloxacin relative to strain G48. However, there was substantial day-to-day variability in uptake kinetics and in the final amount of quinolone accumulated. In parallel experiments, G49 usually accumulated less enoxacin (~73%) than G48 and G49R2. Carbonyl cyanide *m*-chlorophenylhydrazone (20 μM) increased the steady-state concentration of enoxacin and norfloxacin for all strains. In separate experiments, 7.5 mM magnesium chloride and 7.5 mM EDTA decreased accumulation of enoxacin into G48 and G49R2 to a greater degree than in G49 and to concentrations below that accumulated in G49 with magnesium chloride.

Despite repeated attempts, plasmid pNJR3-2 did not trans-

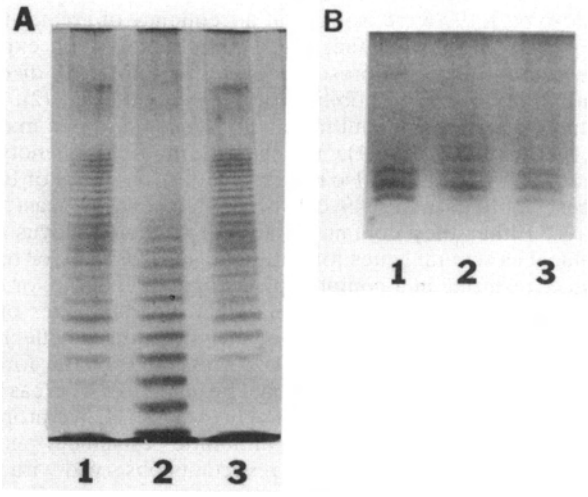

FIG. 2. Silver periodate-stained SDS-polyacrylamide gel electrophoretogram of whole-cell LPS prepared by the method of Hitchcock and Brown (15). (A) Separation on 12% acrylamide gels to emphasize changes in O-antigenic-side-chain-containing LPS. (B) Separation by the method of Lesse et al. (19) to emphasize rough core LPS changes. Lanes 1, G48; lanes 2, G49; lanes 3, G49R2.

fer into G49 by conjugation. Transformation was not performed. Several attempts were also made to mobilize the chromosome of G49 to perform chromosomal mapping; however, despite insertion of the chromosome mobilizing plasmid, R68.45 (32), no DNA could be transferred.

DISCUSSION

The posttherapy isolate G49 had a complex phenotype. In addition to quinolone resistance and reduced susceptibility of cellular DNA synthesis to enoxacin, this strain demonstrated several other changes that could not be explained by a mutation affecting DNA gyrase alone. Even the apparent alteration in the inhibition of DNA gyrase (Table 2) was uncertain, given the pleiotropic cell surface changes. Since these assays were performed in intact cells with or without EDTA, the reduced ability of enoxacin to inhibit DNA synthesis in strain G49 could have reflected a reduced rate of uptake of enoxacin and a reduced effectiveness of EDTA in the ability to permeate the membranes of strain G49 compared with that of strain G48. Strain G49, relative to its paired pretherapy isolate G48, was between 4- and 60-fold more resistant to several β-lactams, chloramphenicol, and tetracycline and had alterations in several outer membrane proteins, including the porin OprF, and in its LPS profile. Furthermore, reversion studies involving isolation of strain G49R2 indicated that these multiple phenotypic changes were somehow genetically linked. Despite enrichment for $OprF^+$ derivatives of G49 by overnight growth on low-salt medium, the finding that all 97 tested $OprF^+$ revertants, including G49R2, had also reverted to the antibiotic susceptibility of the pretherapy isolate appeared inconsistent with two genetically independent mutations.

The ability of such mutants, with multiple changes in antibiotic susceptibility and with multiple phenotypic alterations, to revert in a single step has been documented previously. One genetically well-characterized mutant, an *Escherichia coli* carrying *marA*, has been described in detail by Cohen et al. (8). Such mutants can revert in a single step. Another example is the antibiotic-susceptible mutant Z61 which was isolated in five mutageneses with selection steps. Revertants that had recovered the susceptibility of the wild-type K799 were isolated at a frequency of greater than 10^{-7} (1), despite the fact that subsequent genetic experiments revealed the presence of more than two distinct antibiotic supersusceptibility loci, *absA* and *absB* (2). Another example is the multiresistant *Salmonella typhi* mutant of Gutman et al. (11), which had multiple phenotypic changes. This mutant also reverted with a frequency of 10^{-7}. These mutants and G49 can be explained in at least two ways. Either they contain an altered regulatory locus that influences several genes for antibiotic susceptibility or resistance, resulting in a complex pleiotropic phenotype, or else there is a single dominant mutation which stabilizes other mutations and upon reversion of this mutation the effects of these other mutations are lost. An example of the former type of pleiotropic mutation would be *marA* (8), whereas Z61 has been proposed to fit into the latter class (1). We propose that G49 has a *mar* (multiple antibiotic resistance) phenotype, since none of the partial revertants observed with Z61 (1) were observed for G49.

The mutation in G49 has phenotypic similarities to a mutation in clinical isolate 4419 from a cystic fibrosis patient (6), which lacked OprF and had an apparent DNA gyrase change, although unlike G49, the strain was not cross resistant to non-quinolone antibiotics. In studying strain 4419, Chamberland et al. (6) isolated a spontaneous $OprF^+$ revertant and utilized this to claim that OprF had a null phenotype in this mutant background. However, we feel we must inject two notes of caution into this earlier study. First, it must be pointed out that $OprF^-$ strains grow poorly in vitro and Woodruff and Hancock (42) suggested that their profound structural defects can prevent any conclusive statements about such mutants with respect to antibiotic susceptibility. Second, in this study, a spontaneous $OprF^+$ pseudorevertant, G49R1, which had multiple changes in addition to the obvious change in OprF, was isolated. Such a pseudorevertant was not of great value in interpreting the phenotype of G49.

Despite confusion created by such complex phenotypes of specific posttherapy isolates, it is interesting that $OprF^-$ mutants have been isolated in at least three countries as a result of quinolone therapy (6, 9, 17, 31). These data seem to implicate but certainly do not prove a role for OprF in quinolone susceptibility. It is thus worth considering evidence whether OprF is indeed the major porin in *P. aeruginosa*. This conclusion, which was based originally on studies of mutants and several investigations of model membranes (for a review, see reference 27), was recently challenged on the basis of new model membrane studies by Nakae and collaborators, e.g., Yoshihara and Nakae (44). However, Nikaido et al. (28) have rebutted with the proposal that the model membrane studies of Nakae et al. were incorrectly performed and OprF is indeed the major porin of *P. aeruginosa*. Clearly, the definition of the pathway of uptake across the outer membrane of *P. aeruginosa* cannot be determined precisely in the complex background provided by clinical isolates but awaits isolation of genetically and biochemically defined mutations altering the uptake of quinolones across the outer membrane.

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