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Characterization of Mechanisms of Quinolone Resistance in *Pseudomonas aeruginosa* Strains Isolated In Vitro and In Vivo during Experimental Endocarditis

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Mechanisms of resistance to quinolones were characterized in *Pseudomonas aeruginosa* strains isolated after Tn*5* insertional mutagenesis and in resistant strains that emerged during pefloxacin therapy of experimental aortic endocarditis. Quinolone resistance achieved in in vitro-selected mutants Q^r-1 and Q^r-2 was associated with cross-resistance to several groups of antimicrobial agents, including β-lactams, tetracycline, and chloramphenicol. A significant reduction of norfloxacin uptake was also observed. After ether permeabilization of the cells, DNA synthesis of these two isolates was as susceptible to norfloxacin as DNA synthesis of the parent strain (PAO1). These results indicate that alteration of outer membrane permeability is the primary determinant of resistance in these isolates. This altered cell permeability was correlated with reduction of outer membrane protein G (25.5 kilodaltons) and loss of a 40-kilodalton outer membrane protein in strain Q^r-1. Resistance to quinolones that emerged during experimental endocarditis therapy was associated with both modification of outer membrane permeability (decreased uptake of norfloxacin) and decreased susceptibility of DNA synthesis to norfloxacin. Resistance was limited to quinolones and chloramphenicol. For these strains, norfloxacin inhibitory doses (50%) for DNA synthesis were identical to the drug MICs, suggesting that despite the identification of a permeability change, perhaps due to changes of lipopolysaccharide, the alteration of the quinolone intracellular target(s) susceptibility constitutes the primary determinant of resistance. Also, two distinct levels of norfloxacin resistance of DNA synthesis were found in these isolates, indicating that at least two distinct alterations of the drug target(s) are possible in *P. aeruginosa*.

New fluoroquinolones, such as ciprofloxacin and norfloxacin, have potent antimicrobial activity against gram-positive and gram-negative bacteria, including *Pseudomonas aeruginosa* (48). Biochemical and genetic evidence has designated the DNA gyrase (subunit A) as the intracellular target of these drugs. Bacterial DNA gyrase is a tetramer of two of each of the subunits, A and B, encoded by the *gyrA* and *gyrB* genes, respectively. DNA gyrase catalyzes DNA supercoiling, which serves important functions in DNA replication, recombination, and repair (12). Inhibition of DNA gyrase activities by quinolones leads to inhibition of DNA synthesis, but the mechanisms by which quinolones induce cell death and the exact molecular interactions existing between quinolones, DNA gyrase, and DNA are not clear (24).

Mutational resistance to fluoroquinolones in gram-negative bacteria such as *Escherichia coli* and *P. aeruginosa* has been associated with modification of the intracellular target of the drug (DNA gyrase) or alteration of outer membrane permeability (21, 25, 42, 48).

In *E. coli*, mutations that affect both gyrase subunits, leading to various levels of resistance to fluoroquinolones and nalidixic acid, have been described (24), while in *P. aeruginosa* only one type of gyrase mutation (*nalA* locus) has been identified (22, 26, 42). The outer membrane is the major permeability barrier limiting target access to quinolones and other drugs in gram-negative bacteria. Quinolones can penetrate the outer membrane of *E. coli* not only by diffusion through the OmpF and OmpC porin channels (3, 20, 21, 29) but also by diffusion through the phospholipid layer (3, 5). Therefore, several mutations that modify outer membrane structural components can lead to quinolone resistance. This type of mutation is associated with low-level resistance to quinolones and cross-resistance to other groups of antibiotics that use the same pathways across the outer membrane (48). In *P. aeruginosa*, such broad-spectrum resistance has been described and associated with modification of the outer membrane, such as an apparent acquisition of a 54-kilodalton (kDa) outer membrane protein of an undefined role in resistance (22, 42).

To gain information on the possible mechanisms of resistance to quinolones in *P. aeruginosa*, we have studied the properties of in vitro-isolated quinolone-resistant mutants, as well as the properties of quinolone-resistant *P. aeruginosa* variants isolated from cardiac vegetations during pefloxacin therapy of experimental aortic endocarditis (2).

Tn*5* insertional mutagenesis was used to inactivate or modify existing cellular components to induce quinolone resistance in *P. aeruginosa* PAO1. The resistance achieved in viable mutated strains was associated with cross-resistance to other antimicrobial groups and to reduce uptake of norfloxacin, indicating that cell permeability was altered.

In *P. aeruginosa* strains isolated in vivo, both modification of cell permeability and resistance of DNA synthesis to quinolones were observed. An attempt was made to determine the relative contributions of these factors in the expression of resistance.

In this report, we describe three possibly distinct alterations of outer membrane permeability associated with specific quinolone resistance or broad-spectrum resistance. We

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant properties or genotype	Source (reference)
Bacteria		
Pseudomonas aeruginosa		
PAO1	Wild type	Sokol (46)
Q^r-1	Fluoroquinolone resistant; Tn*5* insertion mutant of PAO1	This study
Q^r-2	Fluoroquinolone resistant; Tn*5* insertion mutant of PAO1	This study
PAO236	PAO1 derivative; *ilv226 his-4 lys-12 met-28 trp-6 proA-82 nalA2*	Haas and Holloway (15)
PAO503	PAO1 derivative; *met-9011*	B. W. Holloway[a]
PA-96	Clinical isolate; fluoroquinolone susceptible	Bayer et al. (2)
$22V_2$	Fluoroquinolone-resistant variant of PA-96, isolated after 4 days of pefloxacin therapy of experimental aortic endocarditis	Bayer et al. (2)
$22V_3$	Same as $22V_2$	Bayer et al. (2)
$23V_1$	Same as $22V_2$ but isolated after 10 days of pefloxacin therapy	Bayer et al. (2)
Haemophilus influenzae Rd	Fluoroquinolone susceptible; used in bioassay	W. L. Albritton[b]
Escherichia coli HB101	F^- *hsd20 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ^-	Maniatis et al. (36)
Plasmid, pUW964	Tn*7*-$Trim^r$ Tn*5*-Neo^r	Sokol (46)

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also describe two distinct levels of resistance of DNA synthesis to norfloxacin in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1.

Drugs. The following antibiotics were used in this study: ciprofloxacin (Miles Laboratory, Rexdale, Ontario, Canada), norfloxacin (Merck Sharp & Dohme Research Laboratory, Rahway, N.J.), enoxacin (Parke-Davis Canada Inc., Toronto, Ontario, Canada), ofloxacin (Ortho Diagnostics, Don Mills, Ontario, Canada), RO 23-6240 (Hoffmann-La Roche Inc., Etobicoke, Ontario, Canada), difloxacin (A-56619) and A-56620 (Abbott Laboratories, Mississauga, Ontario, Canada), cefotaxime and gentamicin (Roussel Canada Inc., Montreal, Quebec, Canada), ticarcillin (Beecham Laboratories, Pointe-Claire, Quebec, Canada), moxalactam and tobramycin (Eli Lilly Canada Inc., Toronto, Ontario, Canada), aztreonam (E. R. Squibb & Sons, Princeton, N.J.), ceftazidime (Ayerst Laboratories, Montreal, Quebec, Canada), cefsulodin (CIBA-GEIGY Corp., Basel, Switzerland), and nalidixic acid, neomycin, tetracycline, and chloramphenicol (Sigma Chemical Co., St. Louis, Mo.).

Media. The following media were used in this study: nutrient broth (BBL Microbiology Systems, Cockeysville, Md.); Mueller-Hinton broth (GIBCO Diagnostics, Madison, Wis.); LB medium, agar, and broth (36); *Pseudomonas* isolation agar (GIBCO); supplemented brain heart infusion agar (GIBCO) as previously described (39); and basic medium [40 mM Tris hydrochloride (pH 7.4), 80 mM potassium chloride, 7 mM magnesium acetate, 2 mM ethyleneglycol-bis-(α-amino-ethyl ether)*N,N'*-tetraacetic acid, 0.4 mM spermidine trihydrochloride, 0.5 M sucrose] (41).

Tn*5* insertion mutagenesis. Tn*5* insertion mutagenesis was performed as described by Sokol (46), with minor modification. Vector pUW964 was used as a suicide plasmid to introduce Tn*5* into *P. aeruginosa* PAO1. The conjugal transfer of pUW964 was done by filter mating of PAO1 and *E. coli* HB101(pUW964). Cells were grown to log phase in LB broth and were mixed in a 2:1 ratio of donor to recipient cells. To select for Tn*5* insertions, the bacteria were grown on *Pseudomonas* isolation agar containing 750 μg of neomycin per ml and incubated for 18 h at 37°C. The bacteria were then plated directly onto *Pseudomonas* isolation agar containing 3 μg of ciprofloxacin per ml. Fluoroquinolone-resistant mutants were isolated at a frequency of 3.5×10^{-11} after 48 h of incubation at 37°C.

DNA probe preparation. A ^{32}P-labeled DNA probe was prepared by using the 3.3-kilobase *Hin*dIII restriction fragment of Tn*5* that contains the neomycin resistance gene. Radioactive labeling of the DNA fragment was performed by nick translation reaction following the recommendation of the *E. coli* DNA polymerase I and DNase I supplier (International Biotechnologies, Inc., New Haven, Conn.) and using [α-^{32}P]dATP (410 Ci/mmol and 10 μCi/μl; Amersham Canada Ltd., Oakville, Ontario, Canada). The probe-specific activity was 3.2×10^7 cpm/μg of DNA.

Hybridization. The presence of the Tn*5* insertion was verified by hybridization of the specific DNA probe to bacterial dots. The bacterial dots were prepared as described by Malouin and Bryan (35). For each bacterial strain tested, 10^7 cells were spotted on a GeneScreenPlus membrane (New England Nuclear Research Products, Lachine, Quebec, Canada). The membrane was prehybridized for 6 h at 65°C, and hybridization was performed for 18 h at 65°C by using 50 ng of denatured Tn*5*-specific probe per ml. Kodak X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) were exposed for 16 h prior to development.

Susceptibility testing. The MICs of drugs were determined by a broth dilution method. The inoculum was 10^5 CFU/ml in a 2-ml final volume of Mueller-Hinton broth. The MIC was defined as the lowest concentration of antibiotic that inhibited the development of visible growth after 20 h at 37°C. The MBCs were determined by plating 0.1 ml from each tube

without visible growth onto Mueller-Hinton agar plates. The MBC was defined as the concentration of antibiotic at which 99.9% of the inoculum was killed at 20 h.

Uptake of norfloxacin. The uptake of norfloxacin by *P. aeruginosa* strains was measured by a modification of the method of Hirai et al. (21, 22). Cells were grown to log phase in nutrient broth with constant agitation at 37°C. Norfloxacin was added to a final concentration of 10 μg/ml, and, at 5 and 30 min of incubation time, 10 ml of the culture was centrifuged at 10,000 × *g* for 3 min. The cells were washed twice in 1 ml of saline and then suspended in 1 ml of saline. This suspension was immersed in boiling water for 7 min to elute norfloxacin and was centrifuged at 10,000 × *g* for 10 min. The activity of norfloxacin in the supernatant was determined by bioassay using *Haemophilus influenzae* Rd grown on supplemented brain heart infusion agar.

The uptake of norfloxacin was also studied in the presence of the energy inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma). This inhibitor was added 5 min before the addition of norfloxacin at a final concentration of 2×10^{-5} M.

The uptake of norfloxacin was measured simultaneously in strains PAO1, Q^r-1, Q^r-2, and PAO236. Five independent uptake experiments were performed, and for each of them four determinations of norfloxacin were done, therefore generating 20 measurements per strain (20 sets of data). The same procedure was used for strain PA-96 and its derivatives, but only two independent uptake experiments were performed, therefore generating eight sets of data. The ratios of the uptake of norfloxacin observed in the fluoroquinolone-resistant derivatives to their parent strains were calculated for each set of data. The means of these ratios were compared with the hypothesized value of one (no differences between the groups) using the hypothesis test for means, mean versus hypothesized value, Mycrostat Program, release 4.1 (Ecosoft Inc., Indianapolis, Ind.).

Norfloxacin inhibition of DNA synthesis. To overcome a possible permeability barrier to norfloxacin and to allow penetration of [^{3}H]dTTP into *P. aeruginosa* cells, DNA synthesis was assayed in permeabilized cells. Bacterial cells were grown to log phase in LB broth at 37°C. The cells were permeabilized by a 45-s ether treatment as described by Rella and Haas (41). The rate of total DNA synthesis was determined by a modification of the method of Rella and Haas (41). The permeabilized cells were diluted (2×10^9 cells per ml) in prewarmed basic medium containing various concentrations of norfloxacin and were incubated at 37°C. At various times, 0.1 ml of the suspension was added to 0.1 ml of prewarmed basic medium supplemented with 80 μM each dATP, dCTP, dGTP, and [^{3}H]dTTP (specific activity, 2.0 Ci/mmol; New England Nuclear Research Products), 0.4 mM NAD, and 4 mM ATP. After 2 min of pulse-labeling at 37°C, the reaction was terminated by adding 3 ml of cold 10% trichloroacetic acid containing 0.05% thymidine and 0.1 M potassium PP_i. The samples were kept on ice for 30 min, and the precipitates were collected on Whatman GFC filters and washed three times with 10% trichloroacetic acid, once with 0.1 M hydrochloric acid, and once with 95% ethanol. The filters were dried and placed in 10 ml of toluene containing 4 g of PPO (2,5-diphenyloxazole) and 0.1 g of dimethyl-POPOP [1,4-bis-(5-phenyloxazolyl)benzene] per liter. The radioactivity was measured by using a Beckman LS-6800 liquid scintillation system. The rate of DNA synthesis was expressed as a percentage of the initial rate at time zero in the absence of drug. The ratio of the rate of DNA synthesis in the presence of norfloxacin to that in the absence of drug was calculated to express the residual DNA synthesis activity.

The norfloxacin 50% inhibitory dose (ID_{50}) was defined as the concentration of drug required to produce 50% of the maximal inhibition of DNA synthesis occurring after 15 or 20 min of incubation in the presence of the drug. Under the conditions used, norfloxacin induced a maximal reduction in DNA synthesis of 50% in all *P. aeruginosa* strains tested, and this was designated as 100% inhibition. Double-logarithmic plots of the relative percentage of inhibition versus the drug concentration were linear ($r > 0.95$) and were used to calculate the ID_{50}.

Isolation and characterization of LPS. Lipopolysaccharide (LPS) was extracted from logarithmic-phase cultures by the method of Darveau and Hancock (9). LPS preparations were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (15%) containing 4 M urea, and visualization was achieved by silver stain (23). The content of 2-keto-3-deoxyoctulosonic acid (KDO) was determined by the method of Karkharis et al. (28) by using the ammonium salt of KDO (Sigma) as a standard.

Isolation and characterization of membrane proteins. Inner and outer membranes of *P. aeruginosa* strains were isolated by the method of Hancock and Nikaido (18), as modified by Godfrey et al. (14). Inner and outer membranes were collected separately and kept frozen at −70°C. Protein concentration was determined by the method of Lowry et al. (33).

Inner or outer membranes (50 μg of protein) were suspended in electrophoretic sample buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris hydrochloride [pH 6.8], 0.002% bromophenol blue). The samples were heated to 100°C for 3 min and were electrophoresed in discontinuous SDS-polyacrylamide gels (12 or 14%) by the method of Laemmli and Favre (30). Gels were stained with 0.1% Coomassie brilliant blue.

Monoclonal antibodies. Monoclonal antibody MCA 48H.3 was used for specific detection of outer membrane protein F of *P. aeruginosa* (13).

Total outer membrane proteins of *P. aeruginosa* PAO1 were electrophoresed as described above, and protein G (25.5 kDa) (16) was electroeluted from SDS-polyacrylamide gel as previously described (43). The protein was acetone precipitated (44) and was found to be free of contaminants when visualized on silver-stained SDS-polyacrylamide gel. A BALB/c mouse (Simonsen Laboratories, Inc., Gibroy, Calif.) was immunized by intraperitoneal injection of 5 μg of purified protein G and 50 μg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Sigma). The same quantity of protein G was injected intraperitoneally once a week for 3 weeks. Subsequently, the mouse was sacrificed and the spleen was removed for fusion. The fusion was performed as described by Longenecker et al. (32) by using the NS1 myeloma cell line. Monoclonal antibody MCA A-20:D-8 was identified by reaction with protein G on Western (immuno-) blot (described below).

Electrophoretic transfer and immunological detection of proteins. After SDS-polyacrylamide gel electrophoresis, outer membrane proteins were transferred to a nitrocellulose membrane by the Western electrophoretic blotting procedure of Towbin et al. (47) 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 37°C in 40 mM Tris hydrochloride (pH 7.5)–50 mM NaCl containing 1% skim milk to saturate additional protein binding sites (27). The monoclonal antibodies were allowed to react for 1 h at 37°C. Peroxidase-conjugated rabbit anti-

TABLE 2. Susceptibilities of *P. aeruginosa* strains to various groups of antibiotics

Antibiotic	MIC[a] (μg/ml) for:						
	PAO1	Q^r-1	Q^r-2	PA-96	$22V_2$	$22V_3$	$23V_1$
Quinolones							
Ciprofloxacin	0.06	0.5	1	0.125	4	2	2
Norfloxacin	0.5	2	8	0.25	16	8	8
Enoxacin	0.5	2	4	0.5	32	16	8
Ofloxacin	0.5	2	4	0.5	16	16	8
RO 23-6240	0.5	4	4	0.5	16	16	8
Difloxacin	2	4	8	0.5	32	32	16
A-56620	0.125	1	1	0.125	8	8	4
Nalidixic acid	32	512	512	64	2,048	2,048	2,048
β-Lactams							
Cefotaxime	16	256	128	16	32	32	64
Ticarcillin	32	128	256	32	32	64	64
Moxalactam	16	64	128	16	16	32	32
Aztreonam	4	32	32	4	4	8	8
Ceftazidime	2	2	2	1	2	4	4
Cefsulodin	2	2	2	1	1	4	2
Aminoglycosides							
Tobramycin	0.25	1	0.5	1	0.5	0.5	0.5
Gentamicin	0.5	2	0.5	1	0.5	0.5	0.5
Others							
Tetracycline	16	128	64	16	16	16	16
Chloramphenicol	16	1,024	128	32	512	256	512

[a] MICs were determined by tube dilution in Mueller-Hinton broth. Strains were incubated for 20 h at 37°C.

mouse antibodies (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) were added, and the color reaction was obtained with Bio-Rad HRP color development reagent according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

Enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay was performed as described by Mackie et al. (34). Outer membrane protein preparations (1 μg per well) were used as the antigen. MCA A-20:D-8 was allowed to react, and peroxidase-conjugated rabbit anti-mouse antibodies were added. The color reaction was obtained by adding hydrogen peroxide and ABTS (2,2′-azino-di-[3-ethyl-benzthiazoline]) as recommended by the supplier (Kirkegaard and Perry Laboratories). The A_{405} was read with an Autoreader (model EL310; Bio-Tek Instruments, Inc., Burlington, Vt.).

Measurement of surface hydrophobicity. Cell surface hydrophobicity was measured by partitioning the bacterial cells between *m*-xylene and 100 mM sodium phosphate (pH 7.2) (5). The index of hydrophobicity (P_s) was expressed as the ratio of decrease in absorbance of the aqueous phase, calculated as (A_{660} of untreated aqueous phase − A_{660} of treated aqueous phase)/A_{660} of untreated aqueous phase.

Lipid extraction. Bacterial cells were grown in nutrient broth to early stationary phase, harvested by centrifugation, washed twice with saline, suspended in water, and freeze-dried. The readily extractable lipid fraction was obtained by chloroform-methanol (2:1) extraction by the method of Folch et al. (11), as modified by Conrad et al. (8). The phosphate content of the readily extractable lipid fraction was determined by the method of Ames and Dubin (1). Phospholipids were resolved by thin-layer chromatography on silica gel G plates (Analtech Inc., Newark, Del.). Chromatograms were developed in chloroform-methanol-acetic acid-water (85:15:10:3.5) (19), dried, and developed in hexane-diethyl ether (4:1) (7). Resolved lipids were visualized with iodine vapor and identified by comigration with standard phospholipids, including lysophosphatidylethanolamine (LPE), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and diphosphatidylglycerol (DPG) (Sigma). Phospholipids were eluted from the silica gel by the method of Skipski et al. (45), and the phosphate content of each of them was determined (1). The molar ratio of phospholipids was calculated assuming 1 mol of phosphate per mol of LPE, PG, and PE and 2 mol of phosphate per mol of DPG. To evaluate the proportion of readily extractable lipid components which do not contain phosphate, immediately after staining with iodine vapor, a Polaroid photograph of the chromatogram was taken and scanned with a model 620 video densitometer (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

RESULTS

Susceptibility of in vitro- and in vivo-selected mutants. After Tn*5* mutagenesis, seven mutants were selected for their ability to grow on *Pseudomonas* isolation agar containing 3 μg of ciprofloxacin per ml. All these isolates showed a strong positive reaction with the Tn*5*-specific DNA probe in a dot hybridization assay. However, we have no evidence that the insertion of Tn*5* caused the resistance observed.

Mutants were divided into two distinct groups according to their susceptibility to norfloxacin. Members of a group are likely siblings. The susceptibilities of two representative isolates (Q^r-1 and Q^r-2) to a broad range of antibiotics are shown in Table 2. Both Q^r-1 and Q^r-2 showed a 16-fold increase in nalidixic acid MICs and an 8-fold increase in A-56620 MICs. Q^r-1 showed a two- to eightfold increase in MICs of all other fluoroquinolones tested, while Q^r-2 was 4 to 16 times more resistant to fluoroquinolones than the parent strain (PAO1). Moreover, the norfloxacin MBC was greater than 32 μg/ml for Q^r-2 but was only 2 μg/ml for Q^r-1 (data not shown).

As shown in Table 2, both mutants were also resistant to other groups of antimicrobial agents. Q^r-1 and Q^r-2 were resistant to β-lactams, such as cefotaxime, ticarcillin, moxalactam, and aztreonam; but neither ceftazidime nor cefsulodin MICs were changed. Q^r-1 also showed a fourfold increase in tobramycin and gentamicin MICs, while Q^r-2 was as susceptible to these agents as the parent strain (PAO1). Finally, both mutants were resistant to tetracycline and chloramphenicol, Q^r-1 being more resistant to these drugs than Q^r-2. Both mutants showed a doubling time of 1 h compared with 0.6 h for PAO1 when incubated in nutrient broth at 37°C.

Susceptibility profiles of three isolates selected during pefloxacin therapy of experimental aortic endocarditis were also studied (Table 2). These isolates were 16 to 64 times more resistant than the parent strain (PA-96) to all quinolones tested, including nalidixic acid. In contrast to in vitro-selected mutants Q^r-1 and Q^r-2, these isolates showed no cross-resistance to the β-lactams or aminoglycosides tested and were not resistant to tetracycline. Cross-resistance to chloramphenicol was also observed in these isolates.

Uptake of norfloxacin. The fact that in vitro-isolated strains Q^r-1 and Q^r-2 were not only more resistant than the parent strain (PAO1) to quinolones but were also resistant to other groups of antimicrobial agents suggested altered membrane permeability. Uptake of norfloxacin was therefore studied in all resistant mutants, as well as in strain PAO236. The latter strain is a well-characterized DNA gyrase mutant (*nalA2*) resistant to quinolones but not to other groups of antimicrobial agents (norfloxacin MIC, 8 μg/ml). The uptakes of norfloxacin by in vitro- and in vivo-selected mutants were compared with the uptakes by the respective parent strains (PAO1 and PA-96) in the presence or absence of CCCP (Fig. 1).

After 30 min of incubation at 37°C with 10 μg of norfloxacin per ml with or without CCCP, drug uptake in Q^r-1 and Q^r-2 was significantly lower ($P < 0.01$) than drug uptake observed in parent strain PAO1 (32 and 13% less uptake, respectively). Although norfloxacin could still associate with the bacterial cells, as seen after 5 min of incubation at 37°C, Q^r-1 and Q^r-2 did not accumulate additional drug between 5 and 30 min of incubation (Fig. 1A). The uptake of ciprofloxacin by PAO503 was also lower than the drug uptake observed in PAO1 (data not shown).

In contrast, norfloxacin uptake in quinolone-resistant strain PAO236 (*nalA2*) was identical to that observed in susceptible strain PAO1.

Norfloxacin uptake was enhanced by 23% after 30 min of incubation in the presence of the energy inhibitor CCCP (20 μM) for strains PAO1 and PAO236, as well as for Q^r-1 and Q^r-2 (Fig. 1A), suggesting that *P. aeruginosa* possesses an active efflux system (6; L. E. Bryan, J. Bedard, S. Wong, and S. Chamberland, Clin. Invest. Med., in press) for fluoroquinolones and that it was intact in the resistant isolates.

As shown in Fig. 1B, the three isolates selected during pefloxacin therapy of experimental aortic endocarditis ($22V_2$, $22V_3$, and $23V_1$) also had a significant reduction ($P < 0.01$) in norfloxacin uptake when compared with parent strain PA-96 (67, 74, and 67% less uptake, respectively). Like Q^r-1 and Q^r-2, these isolates did not accumulate norfloxacin after a 30-min incubation period. CCCP (20 μM) had no significant effect ($P > 0.01$) on strains PA-96 and $22V_2$, while it did enhance norfloxacin uptake by 30% after 30 min of incubation in strain $23V_1$ and by 40% after 5 and 30 min in strain $22V_3$.

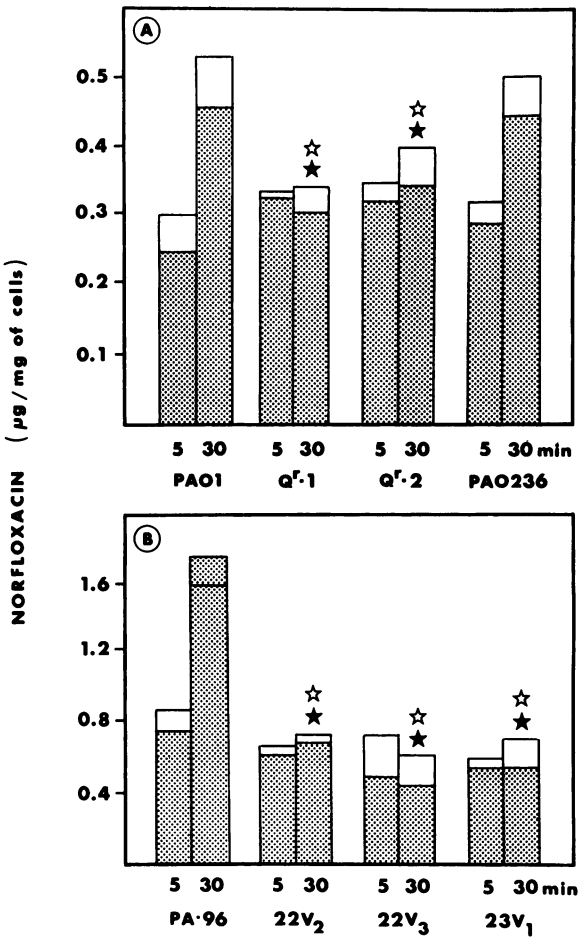

FIG. 1. Uptake of norfloxacin by *P. aeruginosa* PAO1 and laboratory-derived quinolone-resistant strains Q^r-1, Q^r-2, and PAO236 (*nalA2*) (A) and by PA-96 and in vivo-selected resistant mutants $22V_2$, $22V_3$, and $23V_1$ (B). Bacterial cells were incubated in the presence of 10 μg of norfloxacin per ml in nutrient broth at 37°C with constant agitation. Cell-associated norfloxacin was determined after 5 and 30 min of incubation and is expressed as micrograms of drug per milligram (dry weight) of cells. Bars with hatching and open bars represent uptakes of norfloxacin measured in the absence and presence of CCCP (20 μM), respectively. The values represent the means of 20 (A) or 8 (B) determinations. Statistically significant differences ($P < 0.01$) from the mean values obtained for strain PAO1 or PA-96 at 30 min are indicated by closed stars (uptake measured in the absence of CCCP) or open stars (uptake measured in the presence of 20 μM CCCP).

It is of interest to note that strain PA-96 was found to take up 3.2 times more norfloxacin per mg of cells than strain PAO1 and was two times more susceptible to this drug (Table 2). Moreover, the amounts of norfloxacin associated with bacterial cells for strains $22V_2$, $22V_3$, and $23V_1$ were about two times greater than for strains Q^r-1 and Q^r-2. When cell surface hydrophobicity was measured, it was found that the in vivo-selected isolates and their parent strain, PA-96, had a greater cell surface hydrophobicity index (P_s) and therefore were more hydrophobic than in vitro-isolated mutants Q^r-1 and Q^r-2 and parental strain PAO1 (Table 3).

Inhibition of DNA synthesis by norfloxacin. To overcome the previously described permeability barrier to norfloxacin

TABLE 3. Relationship between cell surface hydrophobicity and uptake of norfloxacin

Strain	P_s[a]	Norfloxacin uptake[b] (μg/mg [dry wt] of cells)
PAO1	0	0.54
Q^r-1	0	0.35
Q^r-2	0	0.40
PAO236	0.104	0.50
PA-96	0.355	1.78
$22V_2$	0.264	0.70
$22V_3$	0.245	0.60
$23V_1$	0.391	0.70

[a] The hydrophobicity index is expressed as the partition coefficient in *m*-xylene–100 mM sodium phosphate (pH 7.2).
[b] Norfloxacin uptake after 30 min of incubation at 37°C in nutrient broth containing 10 μg of norfloxacin per ml and 2×10^{-5} M CCCP.

TABLE 4. Inhibition of total DNA synthesis by norfloxacin

Strain	ID_{50}[a] (μg/ml)	MIC[b] (μg/ml)
PAO1	0.31	0.5
Q^r-1	0.31	2.0
Q^r-2	0.24	8.0
PAO503	0.44	1.0
PAO236	8.25	8.0
PA-96	0.23	0.25
$22V_2$	16.00	16.0
$22V_3$	8.25	8.0
$23V_1$	7.25	8.0

[a] The norfloxacin ID_{50} was defined as the concentration of drug required to produce 50% of the maximal inhibition of DNA synthesis after 15 or 20 min of incubation in the presence of drug.
[b] MICs were determined by tube dilution in Mueller-Hinton broth. Strains were incubated for 20 h at 37°C.

in resistant strains and to allow penetration of [^{3}H]dTTP into *P. aeruginosa* cells, the rate of DNA synthesis was measured in ether-permeabilized cells.

The kinetics of inhibition of DNA synthesis by norfloxacin in strain PAO1 is shown in Fig. 2A. In the presence of norfloxacin (0.15, 0.30, and 0.40 μg/ml), there was inhibition of DNA synthesis, the extent of which was dependent on the drug concentration between 15 and 20 min. As described for *E. coli* and *P. aeruginosa* (4, 10), after the initial inhibition the rate of DNA synthesis was resumed, indicating that cellular DNA repair mechanisms responded to the drug-induced damage. The same kinetics of DNA synthesis inhibition by norfloxacin was observed in all *P. aeruginosa* strains studied.

Norfloxacin ID_{50}s were derived from double-logarithmic plots of the relative percentage of inhibition of DNA synthesis versus the drug concentration. An example of such a plot is illustrated for strain PAO1 in Fig. 2B. For every strain, the calculated norfloxacin ID_{50} and the corresponding MIC are given in Table 4. Although in vitro-selected mutants Q^r-1 and Q^r-2 had norfloxacin MICs 4 and 16 times greater than that for the parent strain PAO1, their DNA synthesis was as susceptible to inhibition by the drug. These results indicate that the intracellular target of the drug, the DNA gyrase, was not modified in these isolates.

In contrast, DNA synthesis of strains $22V_2$, $22V_3$, and $23V_1$ was much more resistant to inhibition by norfloxacin than DNA synthesis of parent strain PA-96. Moreover, norfloxacin ID_{50} values determined for these mutants correlated with their MICs. A well-characterized DNA gyrase mutant, PAO236 (*nalA2*), also showed a correlation between the norfloxacin ID_{50} and MIC (Table 4).

Alteration of cell permeability. In an effort to characterize further the alteration of cell permeability leading to resistance to quinolones and other antimicrobial agents in strains Q^r-1 and Q^r-2 and to reduced norfloxacin uptake in strains $22V_2$, $22V_3$, and $23V_1$, several outer membrane and cellular components were examined.

LPS. Analysis of purified LPS electrophoresed on SDS-polyacrylamide gel (15%) containing 4 M urea revealed heterogeneity of O-antigenic side-chain length in the population of LPS molecules isolated from *P. aeruginosa* (9, 40).

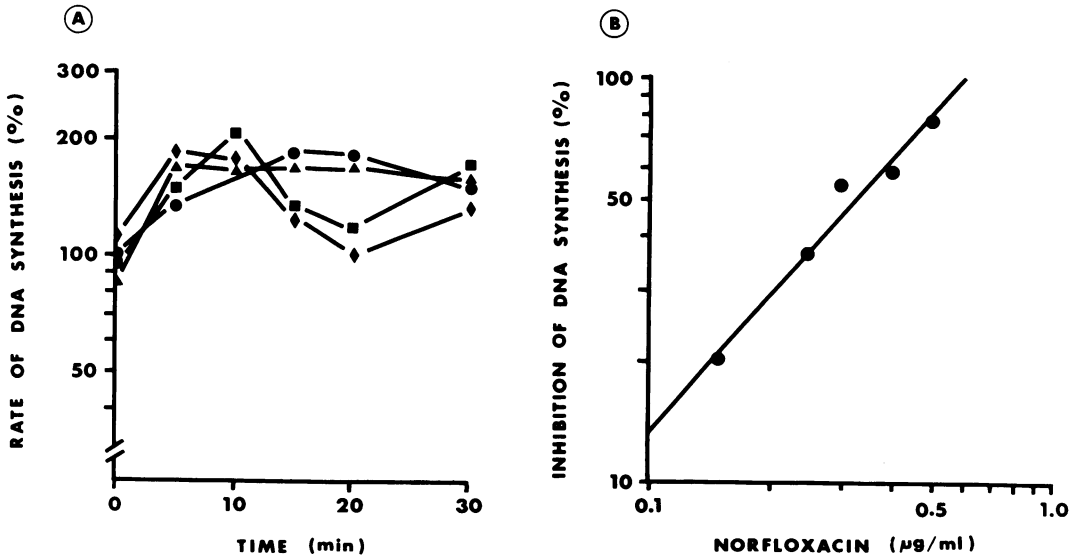

FIG. 2. Inhibition of DNA synthesis by norfloxacin in permeabilized cells of *P. aeruginosa* PAO1. (A) Rate of DNA synthesis without norfloxacin (●) or with 0.15 (▲), 0.30 (■), or 0.40 (◆) μg of norfloxacin per ml. (B) Linear double-logarithmic plot ($r = 0.97$) of the relative percentage of inhibition at 15 min versus the drug concentration used to calculate the norfloxacin ID_{50}.

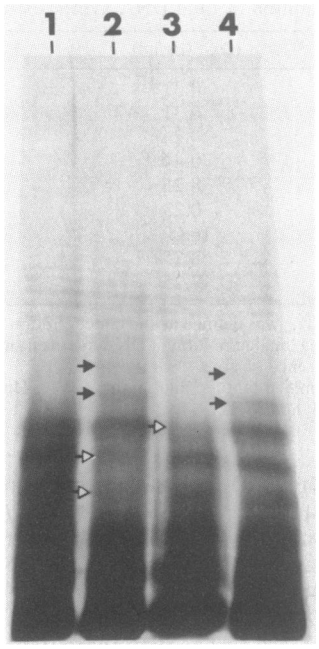

FIG. 3. Silver-stained SDS-polyacrylamide gel (15%) containing 4 M urea of purified LPS. Lanes: 1, PA-96; 2, $22V_2$; 3, $22V_3$; 4, $23V_1$. Arrows indicate changes in LPS banding patterns in the in vivo-selected resistant mutants.

LPS banding patterns of in vivo-selected mutants $22V_2$, $22V_3$, and $23V_1$ were compared with the LPS banding pattern of PA-96 (Fig. 3). Strains $22V_2$ and $23V_1$ (Fig. 3, lanes 2 and 4) showed two additional LPS bands of medium length that were not present in the parent strain (Fig. 3, lane 1). This was also combined with the marked reduction of two LPS bands of short length in isolate $22V_2$. A marked reduction of an LPS band of short length was also observed in strain $22V_3$ (Fig. 3, lane 3). The KDO contents of the LPS were the same in strain PA-96 and the mutants (50 μg/mg of LPS [dry weight]). This suggests that the LPS core remained unchanged in these resistant isolates.

LPS isolated from quinolone-resistant isolates Q^r-1 and Q^r-2 was not different from LPS isolated from the parental strain PAO1, based on visualization of LPS on silver-stained SDS-polyacrylamide gel (data not shown) and determination of KDO content (55 μg/mg of LPS [dry weight]).

Inner membrane proteins. Inner membrane protein profiles were visualized on Coomassie blue-stained SDS-polyacrylamide gel (12%). These profiles were identical for all resistant isolates and parent strains studied (data not shown).

Outer membrane proteins. Outer membrane proteins were separated by electrophoresis in SDS-polyacrylamide gel (10 to 14%), and the protein profiles were visualized by Coomassie blue staining.

Outer membrane protein profiles obtained for strains $22V_2$, $22V_3$, and $23V_1$ were identical to that of strain PA-96. By using a protein F-specific monoclonal antibody (MCA 48H.3), two protein bands (35 and 39 kDa) were visualized on immunostained Western blot for each of these isolates (data not shown). This observation indicates that the cause of altered membrane permeability leading to reduced uptake of norfloxacin in these mutants did not reside in major rearrangements of the outer membrane protein or modification in protein F.

Outer membrane protein profiles obtained from strains PAO1, Q^r-1-type derivatives, and Q^r-2-type derivatives are shown in Fig. 4 (lanes 1, 6 to 8, and 2 to 5, respectively). A 25.5-kDa outer membrane protein, identified as protein G (16), was in significantly lower quantity in the outer membrane of Q^r-1-type mutants (Fig. 4, lanes 6 to 8). By using MCA A-20:D-8, two protein bands (25.5 and 40 kDa) were visualized on an immunostained Western blot of the outer membrane protein profile of PAO1 (Fig. 5B, lane 1). MCA A-20:D-8 did not detect the 40-kDa outer membrane protein in the Q^r-1 mutant (Fig. 5B, lane 2). A laboratory-derived mutant of PAO1, PAO503, also showed a similar modification of protein G (Fig. 5A and B, lanes 4). These changes were not present in isolate Q^r-2. No changes in the major outer membrane protein, protein F, could be seen on Coomassie-stained SDS-polyacrylamide gel (Fig. 4) or visualized on MCA 48H.3 immunostained Western blot (Fig. 5C) in strains Q^r-1, Q^r-2, and PAO503.

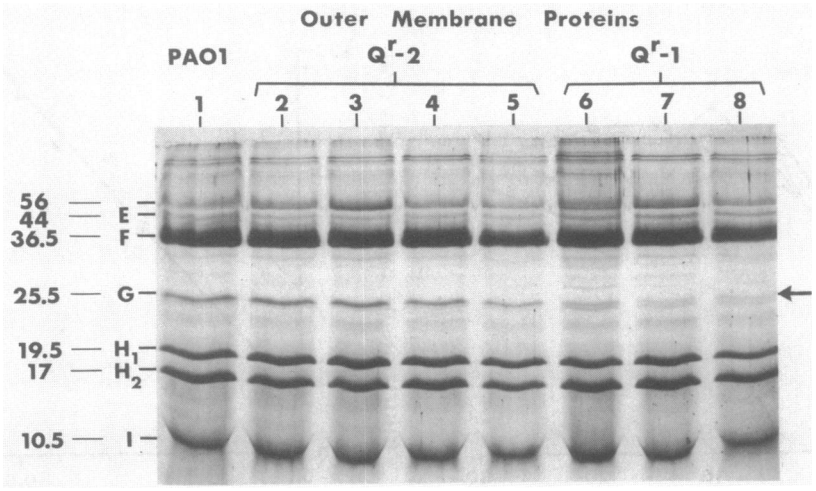

FIG. 4. Polyacrylamide gel (14%) electrophoresis of outer membrane proteins isolated from PAO1 and fluoroquinolone-resistant derivatives isolated after Tn*5* mutagenesis. Lanes: 1, PAO1; 2 to 5, group of mutants showing a 16-fold increase in the MIC of norfloxacin; 6 to 8, group of mutants showing a 4-fold increase in the MIC of norfloxacin. Representative isolates Q^r-1 and Q^r-2 are shown in lanes 4 and 7, respectively. On the left are indicated the major outer membrane proteins of *P. aeruginosa* and their corresponding molecular weights (10^3). The arrow indicates the modification of protein G observed in Q^r-1-type mutants.

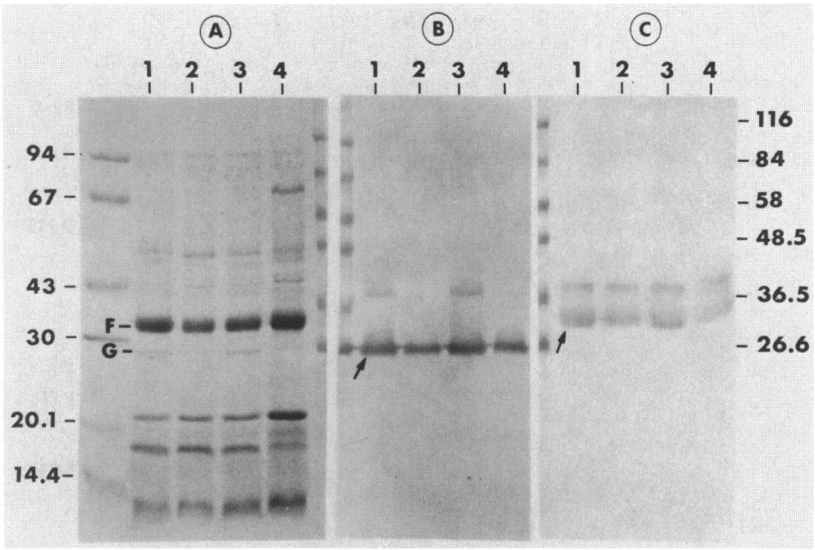

FIG. 5. (A) Polyacrylamide gel (gradient, 10 to 12%) electrophoresis of outer membrane proteins isolated from bacterial cells grown in Mueller-Hinton broth. F indicates the position of protein F (35.5 kDa), and G indicates the position of protein G (25.5 kDa). (B) Western blot of a replicate of the polyacrylamide gel shown in panel A immunostained by using monoclonal antibody MCA A-20:D-8. The arrow indicates the specific reaction in PAO1. The reaction was allowed to proceed for 15 min to maximize the difference observed in the 40-kDa protein. (C) Western blot of a replicate of the polyacrylamide gel shown in panel A immunostained by using protein F-specific monoclonal antibody MCA 48H.3. The arrow indicates the protein F-specific reaction in PAO1. Lanes in panels A, B, and C are as follows: 1, PAO1; 2, Q^r-1; 3, Q^r-2; 4, PAO503. Numbers on the left and right indicate molecular weights (10^3) of standard proteins.

MCA A-20:D-8 was also used in a direct enzyme-linked immunosorbent assay to quantify the degree of reduction of protein G in the outer membrane. Strains Q^r-1 and PAO503 showed 80 and 60% less protein G in their outer membranes, respectively, compared with the amount present in the outer membrane of PAO1.

Q^r-1 was two times more resistant to norfloxacin than PAO503, which in turn was two times more resistant to the drug than PAO1. The susceptibilities of DNA synthesis to norfloxacin in these three strains were equivalent (Table 4). PAO503 also consistently showed a twofold increase in ciprofloxacin, enoxacin, A-56620, and nalidixic acid MICs. Cross-resistance to tetracycline and chloramphenicol was also observed in this strain (two- and fourfold increases in MIC, respectively). A correlation can be proposed between reduction of protein G in the outer membrane and altered cell permeability leading to increased resistance to norfloxacin in *P. aeruginosa*.

Phospholipids. The phospholipid composition of *P. aeruginosa* whole cells was visualized by thin-layer chromatography (Fig. 6). There was no difference of phospholipid composition between in vitro- or in vivo-selected quinolone-resistant mutants and their respective parental strains. The ratio of free fatty acids determined by scanning densitometry was evaluated at 20 and 15% in PAO1 and PA-96, respectively, and was also unchanged in the resistant mutants.

DISCUSSION

Our studies on quinolone resistance in laboratory-derived mutants and in vivo-selected isolates have led to the proposition that there are at least two, and possibly three, mechanisms by which entry of norfloxacin and other quinolones can be reduced in cells.

Recently, it was proposed that quinolones can cross the outer membrane by diffusion (3) involving both porin channels (20, 21, 25) and the LPS:phospholipid bilayer (5).

In strains Q^r-1 and Q^r-2, quinolone resistance was associated with resistance to other antimicrobial agents, such as certain β-lactams, tetracycline, and chloramphenicol (Table 2). Both strains showed a reduction of norfloxacin uptake compared with their parent strain, PAO1 (Fig. 1). Cross-resistance with antibiotics known to use the porin pathway across the outer membrane (i.e., β-lactams) (29, 38) and the slower growth rate of these mutants indicated that the alteration of cell permeability was likely to result from modification of porin channels.

Although it has been shown that protein F forms porin channels in the outer membrane of *P. aeruginosa* (17), mutants lacking protein F were not isolated in this study.

Instead, alteration of membrane permeability in isolate Q^r-1 was associated with a reduction of the quantity of protein G (25.5 kDa) (Fig. 4) and a complete loss of an antigenically related 40-kDa outer membrane protein identified by using a monoclonal antibody (Fig. 5B). The structural or functional relationship between these two outer membrane proteins remains unclear. The 40-kDa protein was distinct from the two forms of protein F (35 and 39 kDa) identified with a monoclonal antibody (Fig. 5C). At present, the function of protein G in the outer membrane of *P. aeruginosa* is unknown.

It is of interest to note that independently isolated strain PAO503, exhibiting low-level fluoroquinolone resistance and cross-resistance to tetracycline and chloramphenicol, presented a similar modification in outer membrane protein profile. Recently, ciprofloxacin-resistant *P. aeruginosa* derivatives were isolated during ciprofloxacin therapy of patients with cystic fibrosis. These isolates were shown to have a 90% decrease of protein G in their outer membrane, which was also coupled to the loss of the 40-kDa protein (H. R.

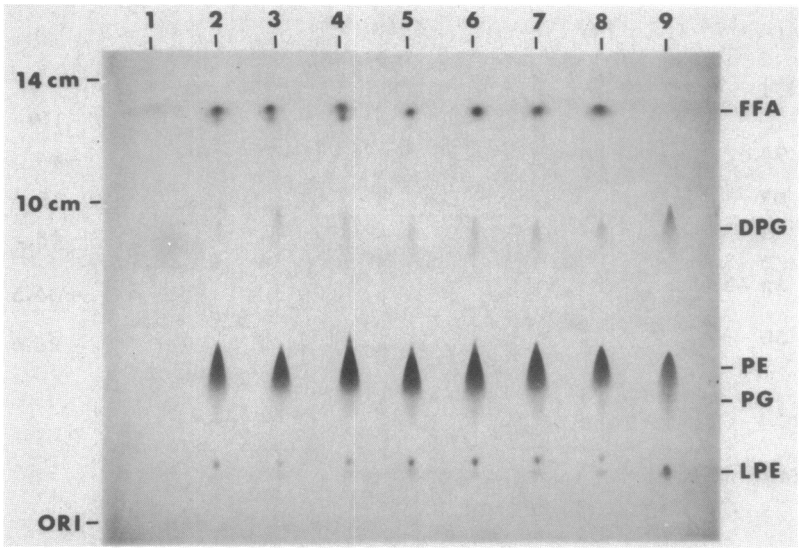

FIG. 6. Thin-layer chromatography analysis of phospholipids isolated from freeze-dried, whole bacterial cells. ORI indicates where the readily extractable lipid samples were applied. The chromatogram was developed to a distance of 10 cm in chloroform-methanol-acetic acid-water (85:15:10:3.5) and to a distance of 14 cm in hexane-diethyl ether (4:1). Identification of visualized lipids was done by comigration with standards. Lanes: 1, free fatty acid (FFA) standard ($C_{17:0}$ heptadecanoic acid); 2, PAO1; 3, Q^r-1; 4, Q^r-2; 5, PA-96; 6, $22V_2$; 7, $22V_3$; 8, $23V_1$; 9, phospholipid standards mixture: diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and lysophosphatidylethanolamine (LPE).

Rabin and L. E. Bryan, personal communication). This substantiates a role for this outer membrane modification in the expression of fluoroquinolone resistance and indicates that this mechanism is also clinically relevant.

Since a reduced copy number of protein G in the outer membrane correlated with decreased entry of drug and resistance to several antibiotic groups, we suggest that protein G has porin channel-forming activity or that reduction of this protein and loss of the 40-kDa protein would alter protein F pore function, perhaps through a change in outer membrane stability. These hypotheses are under investigation.

Protein G and the 40-kDa protein are unlikely to be norfloxacin-binding proteins, since initially (at 5 min) cell-associated norfloxacin was not reduced in strain Q^r-1 (Fig. 1). A previous report related permeability-mediated resistance to norfloxacin to the acquisition of a new outer membrane protein (54 kDa) (22). Such a change was not observed in the outer membranes of *P. aeruginosa* isolates studied here. This report is the first implicating reduction of protein G as a possible mechanism of outer membrane permeability modulation. However, since protein G is not a major outer membrane protein and the change observed is not a complete loss of the protein, such modification may have been overlooked by other investigators. In fact, in the report describing a new 54-kDa outer membrane protein (22) it also appears that protein G may have been reduced in quantity.

In reconstitution studies using lipid bilayers, purified *P. aeruginosa* protein F was shown to form very few large porin channels but a large number of very narrow channels (49). An exclusion of >3 kDa was attributed to the large channels (17), while the small pores prevented β-lactams from entering the bacterial cells (37). Conformational changes of protein F could affect the proportion of large and small pores in the outer membrane and modulate membrane permeability.

It was previously proposed that modified configuration of protein F, induced by a single- or possibly double-amino-acid change, decreased β-lactam permeation through the outer membrane of *P. aeruginosa* (13). Such functional changes in protein F could be at the origin of the permeability-mediated resistance observed in strain Q^r-2 and would not be detectable by the approaches used in this study.

LPS changes have been correlated with reduced uptake of norfloxacin in strains $22V_2$, $22V_3$, and $23V_1$ (Fig. 3). Recently, it was proposed that quinolones interact with the outer membrane of *E. coli* as chelating agents (5). They would create displacement of membrane-bound Mg^{2+}, exposing hydrophobic domains on the outer membrane through which they would penetrate the bacterial cells. Therefore, quinolones may be able to penetrate the outer membranes of all gram-negative bacteria by this additional nonporin pathway (5). The outer leaflet of the outer membrane phospholipid bilayer contains anionic LPS molecules stabilized by divalent cations such as Mg^{2+} (31). LPS changes observed in the in vivo-selected resistant mutants may reflect a change in divalent cations' saturation of the outer membrane that would in turn affect the nonporin pathway of quinolone uptake. However, mutation(s) rendering DNA synthesis more resistant to quinolones would be the primary determinant of resistance in these isolates. Even though reduced uptake of norfloxacin was demonstrated for these three strains, altered cell permeability would contribute modestly to the expression of quinolone resistance in these isolates.

Alteration of outer membrane permeability leading to drug resistance has sometimes been associated with modification of phospholipid composition, in particular for antibiotics interacting with components of the outer membrane, such as polymyxin B and aminoglycosides (7, 8). No such changes were identified in this study (Fig. 6).

Quinolones are also actively pumped out of bacterial cells (6; Bryan et al., in press). The activity of the efflux system was unaltered in the quinolone-resistant mutants studied.

Resistance to quinolones may also be achieved by mutational modification of the intracellular target of the drug, the DNA gyrase. To determine whether such a mechanism of

resistance was detected in *P. aeruginosa* strains studied, the susceptibility of DNA synthesis to norfloxacin was used as an indirect measurement of the susceptibility of DNA gyrase to this drug. DNA synthesis was found to be more resistant to inhibition by norfloxacin only in the strains isolated during pefloxacin therapy of experimental endocarditis (Table 4). Norfloxacin ID_{50} values clearly showed two levels of DNA synthesis resistance to the drug (8 and 16 μg/ml). This indicates that we have isolated at least two different DNA gyrase mutations. Both mutations rendered the bacterial cells resistant to all fluoroquinolones tested, as well as to nalidixic acid.

For the in vitro-selected mutants, the primary mechanism of quinolone resistance was found to be alteration of outer membrane permeability reducing drug entry, probably owing to changes in porin channels. This type of resistance was associated with resistance to other antimicrobial agents. None of the quinolone-resistant mutants isolated in vitro was resistant due to a DNA gyrase mutation. Although we have no evidence that insertional mutagenesis is the cause of permeability-mediated ciprofloxacin resistance in Q^r-1 and Q^r-2, the low frequency of isolation of mutants may indicate that inactivation of DNA gyrase genes (*gyrA* or *gyrB*) by insertion of Tn*5* is a lethal event for the bacteria, due to the role of gyrase in critical cellular functions.

In isolates selected during pefloxacin therapy of experimental aortic endocarditis (2), a mutation(s) rendering DNA gyrase more resistant to quinolones was found to be the primary determinant of resistance, even though altered permeability associated with changes in LPS was also observed. It is of interest to note that these mutants are not resistant to other antibiotics except chloramphenicol. The resistance to chloramphenicol is unlikely to be explained by acquisition of R factor, since we were unable to detect any plasmid DNA (data not shown). Mutants with the same permeability defect as the in vitro-isolated resistant strains were not isolated during experimental therapy of *P. aeruginosa* endocarditis. Altered permeability confers a relatively low level of resistance to highly potent quinolones (Table 2). Such mutants would be selected against in an environment where the drug concentration can exceed the low level of resistance produced only by permeability changes.

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