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Resistance to Pefloxacin in *Pseudomonas aeruginosa*

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Mechanisms of resistance to pefloxacin were investigated in four isogenic *Pseudomonas aeruginosa* strains: S (parent isolate; MIC, 2 μg/ml), PT1 and PT2 (posttherapy isolates obtained in animals; MICs, 32 and 128 μg/ml, respectively), and PT2-r (posttherapy isolate obtained after six in vitro subpassages of PT2; MIC, 32 μg/ml). [2-^{3}H]adenine incorporation (indirect evidence of DNA gyrase activity) in EDTA-permeabilized cells was less affected by pefloxacin in PT2 and PT2-r (50% inhibitory concentration, 0.27 and 0.26 μg/ml, respectively) than it was in S and PT1 (50% inhibitory concentration, 0.04 and 0.05 μg/ml, respectively). Reduced [^{14}C]pefloxacin labeling of intact cells in strains PT1 and PT2 correlated with more susceptibility to EDTA and the presence of more calcium ($P < 0.05$) and phosphorus in the outer membrane fractions. Outer membrane protein analysis showed reduced expression of protein D2 (47 kDa) in strains PT1 and PT2. Other proteins were apparently similar in all strains. The addition of calcium chloride (2 mM) to the sodium dodecyl sulfate-solubilized samples of outer membrane proteins, before heating and Western blotting, probed with monoclonal antibody anti-OmpF showed electrophoretic mobility changes of OmpF in strains PT1 and PT2 which were not seen in strain S. Calcium-induced changes were reversed with ethyleneglycoltetraacetate. Decreased [^{14}C]pefloxacin labeling was further correlated with an altered lipopolysaccharide pattern and increased 3-deoxy-D-mannooctulosonic acid concentration ($P < 0.01$). These findings suggested that resistance to pefloxacin is associated with altered DNA gyrase in strain PT2-r, with altered permeability in PT1, and with both mechanisms in PT2. The decreased expression of protein D2 and the higher calcium and lipopolysaccharide contents of the outer membrane could be responsible for the permeability deficiency in *P. aeruginosa*.

The activities of fluoroquinolones against *Pseudomonas aeruginosa* have been fully documented (45), but bacteria can develop resistance to these agents (12, 18, 19, 36, 38). This resistance results from reduced permeability of the outer membrane (OM), altered DNA gyrase, or a combination of the two processes (7, 8, 18, 36, 38). However, mechanisms of resistance at the molecular level are not yet entirely elucidated (45).

In a pseudomonal peritonitis model, we showed previously that resistance emerges rapidly when mice are treated with pefloxacin or ciprofloxacin (26). In mice infected with a quinolone-susceptible *P. aeruginosa* isolate, posttherapy variants (strain PT1) emerged after a single dose of pefloxacin, showing an 8- to 16-fold decrease in susceptibility to quinolones. Subinoculation of a PT1 strain, followed by therapeutic exposure to ciprofloxacin, produced a highly resistant posttherapy variant (strain PT2) for which the MIC was increased 64-fold (26).

We report here results of further studies on the mechanism of resistance of these strains. As expected, we observed involvements of DNA gyrase and permeability, but we were surprised by the complexity of the OM changes in the latter case.

MATERIALS AND METHODS

Bacterial strains. Four isogenic strains of *P. aeruginosa* 305 were used in this study. Strain S is a clinical isolate (pefloxacin MIC, 2 μg/ml); posttherapy strain PT1 (pefloxacin MIC, 32 μg/ml) was obtained from a mouse that was infected with S and treated with a single dose of pefloxacin as described previously (26); posttherapy strain PT2 (pefloxacin MIC, 128 μg/ml) was obtained from another mouse that was infected with PT1 and treated with five doses of ciprofloxacin (26). Strain PT2-r (pefloxacin MIC, 32 μg/ml) was obtained after six subpassages of strain PT2 on antibiotic-free nutrient agar. The four strains were identical according to biochemical identification with the API system (Appareil et Procédés d'Identification, International S.A., Geneva, Switzerland) and serotyping (O:11) by the International Antigen Typing Scheme for *P. aeruginosa*. Stock cultures were maintained in skim milk at −70°C. At the start of the experiments, strains were thawed, grown in 5 ml of either Mueller-Hinton broth (Oxoid, United Kingdom) or M9 medium (28), and incubated overnight at 37°C. The susceptibilities of the four strains to pefloxacin were regularly controlled, as follows (27). A total of 100 μl of overnight culture, i.e., about 2×10^8 to 3×10^8 CFU, was uniformly spread over square Mueller-Hinton agar plates containing a concentration gradient of pefloxacin. After incubation, the confluent growth observed at the lower antibiotic concentrations was limited by a sharp boundary corresponding to the MIC.

Chemicals. Unlabeled pefloxacin and [^{14}C]pefloxacin (19 mCi/mmol; C.E.N. Saclay, Gif-sur-Yvette, France) were kindly provided by Rhône-Poulenc, Paris, France. [2-^{3}H]adenine (24 Ci/mmol) was purchased from Amersham International plc, Buckinghamshire, England. Other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

DNA inhibition synthesis. Intact cells were incubated for 10 min at 37°C in the presence of various concentrations of pefloxacin. Then, uptake of [2-^{3}H]adenine was determined as described by Benbrook and Miller (3). Some experiments were carried with EDTA-permeabilized cells (6), except that incubation in the presence of 0.2 mM EDTA was limited to 1 min (instead of 2 min in the original method used for *Escherichia coli*).

[^{14}C]pefloxacin labeling of whole cells. Prewarmed flasks containing 300 ml of Mueller-Hinton broth were inoculated with 3 ml of an overnight preculture and incubated at 37°C

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with rotatory shaking (350 rpm). Cells were harvested at the mid-exponential phase of growth (optical density at 650 nm, 0.6 to 0.7) by centrifugation at 5,000 × *g* for 20 min at 20°C and resuspended in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (10 mM, pH 7.3) at a cell density of 16 ± 0.9 mg (wet weight)/ml (1.46 ± 0.19 mg [dry weight]/ml). A vial containing 450 μl of a bacterial suspension ($1.76 \times 10^9 \pm 0.16 \times 10^9$ CFU) was incubated at 30°C under shaking, and at time zero, 50 μl of [^{14}C]pefloxacin solution (final concentration, 2.5 μg/ml) was added. At various times thereafter, 50 μl of unlabeled pefloxacin (final concentration, 1 mg/ml) was added to the mixture, and the whole volume was immediately filtered, under vacuum, through two superimposed glass microfiber filters (Whatman Ltd., Maidstone, United Kingdom) which were prewetted with unlabeled pefloxacin. Filters were rapidly washed twice with 2 ml of 0.9% NaCl, dried, and placed into 2 ml of Lumagel SB (Lumac/3M bv, Schaesberg, The Netherlands); and the radioactivity on the filters was counted. The radiolabeled pefloxacin that bound to filters without bacteria was measured and subtracted from the results. Experiments were run in parallel with the four strains, and three measurements were made for each time of incubation. Results are expressed as mean values of four to eight independent experiments.

The effect of carbonyl cyanide chlorophenylhydrazone (CCCP) at a final concentration of 100 μM was determined by adding this product 5 min after the addition of [^{14}C]pefloxacin.

Other assays were carried out after a 3-min pretreatment with EDTA (1 mM), calcium chloride (10 mM), or magnesium chloride (10 mM).

EDTA susceptibility. Mid-exponential-phase cultures in Mueller-Hinton broth were harvested by centrifugation at 20°C, and cells were resuspended in 10 mM MOPS buffer (pH 7.3). EDTA was added at a final concentration of 1 mM, and the optical density at 650 nm was monitored for up to 20 min at room temperature. Control experiments without EDTA were run simultaneously.

OM and LPS preparation. Cell envelopes and OMs were prepared as described by Spratt (40) and Marchou et al. (25). The amphoteric MOPS buffer solution was used to avoid interference with the divalent cation composition of the cell wall. Cells were grown and harvested as described above, washed once with 30 mM MOPS–200 mM NaCl buffer (pH 8.0), and resuspended (100 mg [wet weight]/ml) in the same buffer containing 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 10 mM 2-mercaptoethanol. Cells were sonicated by three 30-s bursts at an output of 5 and a duty cycle of 90% (Branson sonifier; Branson Instruments, Danbury, Conn.). Membranes were pelleted by centrifugation at 110,000 × *g* for 50 min and washed twice with 15 mM MOPS–100 mM NaCl (pH 8.0). Membrane suspensions were then incubated with 2% (wt/vol) Sarkosyl NL-97 detergent at room temperature for 20 min. The insoluble OM fraction was pelleted by centrifugation at 40,000 × *g* at 10°C, resuspended in distilled water at a protein concentration of about 10 mg/ml, and stored at −20°C. Protein concentrations were determined by the BCA protein assay (Pierce Chemical Co., Rockford, Ill.). Bovine serum albumin was used as a standard.

Lipopolysaccharide (LPS) fractions were prepared from mid-exponential-phase cultures as described by Darveau and Hancock (13) and subjected to chloroform-methanol extraction (1:1; vol/vol).

Gel electrophoresis and immunoblotting. OM proteins (OMPs) and LPS were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a Protean II Slab Electrophoresis Cell (Bio-Rad Laboratories, Richmond, Calif.). OMP and LPS fractions were solubilized in sample buffer and heated at 95°C for 5 min. OMP samples were electrophoresed on 10% acrylamide resolving gel, and LPS samples were electrophoresed on 14% acrylamide and 4 M urea. Electrophoresis was carried out at a constant current of 10 mA per gel. Silver staining, after treatment of the gels with glutaraldehyde (32), was used to detect both proteins and lipids. Carbohydrate-specific silver staining of LPS was performed by potassium dichromate oxidation with Bio-Rad reagents.

For OmpF immunoblotting, OMPs separated by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane (0.45-μm pore size in roll form; Millipore Corp., Bedford, Mass.) for 2 h in a Bio-Rad Mini Trans Blot Electrophoretic Transfer Cell containing 25 mM Tris hydrochloride (pH 8.3), 192 mM glycine, and 20% (vol/vol) methanol. Immunoblotting was performed as described by Towbin et al. (42) by using monoclonal antibody (MA5-8) anti-*P. aeruginosa* porin F (30), which was kindly provided by W. A. Woodruff and R. E. W. Hancock (Vancouver, British Columbia, Canada). Bound antibody was detected with rabbit anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands) by using 4-chloro-1-naphthol and hydrogen peroxide as reagents.

OM ion analysis. The divalent cation content of OMs was determined with a Perkin-Elmer model 430 Atomic Absorption Spectrophotometer (Ueberlingen, Germany). Standards were made by using a standard electrolyte solution (Titrisol; Merck Co., Darmstadt, Germany). The phosphorus content was measured by the method of Stanton (41). Specimens were diluted to fit within the linear range of standard curves for calcium, magnesium, or phosphorus. Results are given as micrograms of divalent cation or phosphorus per milligram of protein.

KDO assay. 3-Deoxy-D-mannooctulosonic acid (KDO) was determined by the colorimetric assay of Karkhanis et al. (20) on OM samples hydrolyzed in 0.2 N H_2SO_4 for 25 min at 100°C. The ammonium salt of KDO was used as the standard.

RESULTS

DNA synthesis inhibition by pefloxacin. As reported previously (1), *P. aeruginosa* does not incorporate thymidine, so we used [2-^{3}H]adenine to measure the effect of pefloxacin on DNA synthesis. The 50% inhibitory concentration calculated from the data in Fig. 1A were 2.64, 29.25, 20.08, and >100 μg/ml for strains S, PT1, PT2-r, and PT2, respectively. However, in control experiments without pefloxacin, strains S and PT2-r incorporated 2.5-fold more adenine than did PT1 and PT2. We suspected that cell permeability to [2-^{3}H]adenine could be affected in strains PT1 and PT2. In order to overcome this barrier, cells were pretreated with 0.2 mM EDTA for 1 min. This resulted in equalization of [2-^{3}H]adenine incorporation without pefloxacin and without the loss of cell viability. In EDTA-permeabilized cells (Fig. 1B), DNA synthesis became very susceptible to pefloxacin; and the 50% inhibitory concentrations decreased to 0.04, 0.05, 0.25, and 0.27 μg/ml for strains S, PT1, PT2-r, and PT2, respectively. Assay results were similar for strains S and PT1, on the one hand, and for strains PT2 and PT2-r, on the other.

[^{14}C]pefloxacin labeling of whole cells. In preliminary ex-

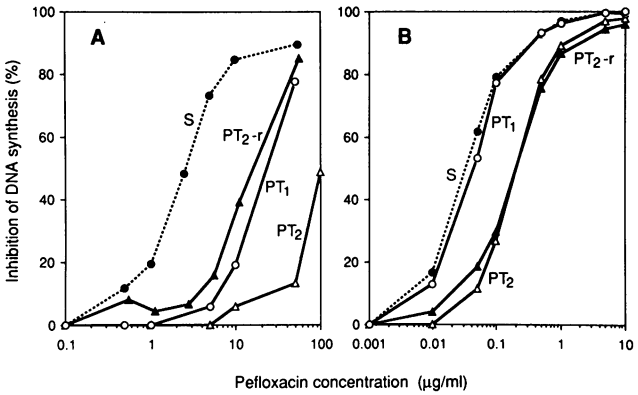

FIG. 1. Inhibition of DNA synthesis by pefloxacin in intact cells (A) and EDTA-permeabilized cells (B) of quinolone-susceptible and quinolone-resistant *P. aeruginosa* strains. Standard conditions were used, as described in the text.

periments, the pH dependence of [^{14}C]pefloxacin uptake was measured from pH 6.5 to 8.5 in 10 mM MOPS buffer at 30°C. The uptake peaked at between pH 7.0 and 7.5 and decreased sharply at pH 8.0 (data not shown). Therefore, the following experiments were carried out at pH 7.3.

[^{14}C]pefloxacin accumulation by bacterial cells was unsaturable when the drug concentration varied from 10 to 100 μg/ml. Two patterns of labeling were observed during the first 5 min of incubation. Strains S and PT2-r bound more radioactivity than did strains PT1 and PT2 (Fig. 2). Looking carefully at the initiation of labeling, we noticed an unexplained but regular drop after 15 to 30 s of incubation in strain S but not in the other three strains. For all strains, a steady state (plateau) occurred between minutes 5 and 30 (data not shown). When CCCP was added after 5 min of incubation, [^{14}C]pefloxacin labeling increased again (Fig. 2). This CCCP-induced increase of labeling was measurable 30 s after its addition, and in PT2 cells, labeling dropped slightly before the onset of the increase. Five minutes after the addition of CCCP (i.e., after 10 min of incubation overall),

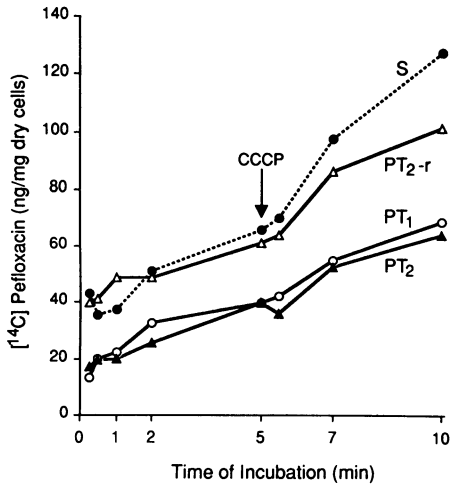

FIG. 2. Labeling of quinolone-susceptible and quinolone-resistant *P. aeruginosa* strains by [^{14}C]pefloxacin and the effect of CCCP (100 μM) added at the time indicated by the arrow. Standard conditions were used, as described in the text.

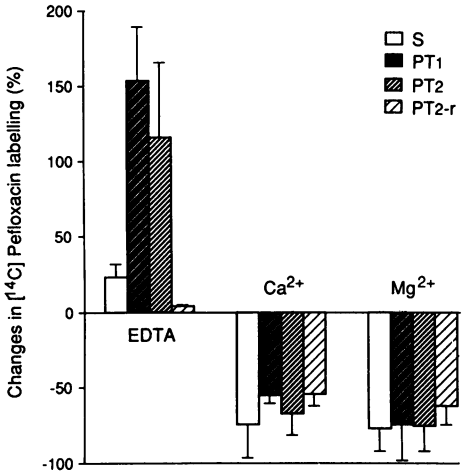

FIG. 3. Effect of EDTA (1 mM), calcium chloride (10 mM), and magnesium chloride (10 mM) on [^{14}C]pefloxacin labeling of quinolone-susceptible and quinolone-resistant *P. aeruginosa* strains.

cells from the four strains bound 60 to 80% more radioactivity than the CCCP-free controls did (data not shown), whereas the relative difference in labeling between the strains remained similar. When bacterial cells were permeabilized by EDTA pretreatment (Fig. 3), radiolabeling (as measured after 5 min of incubation with [^{14}C]pefloxacin) was enhanced by less than 25% in strains S and PT2-r and more than 100% in strains PT1 and PT2 ($P < 0.05$). Assays were also carried out with strains S and PT1 incubated first with [^{14}C]pefloxacin for 5 min and then with 1 mM EDTA for 1 min. This resulted in a 30% increase in cell labeling with strain PT1, but no changes were observed with strain S. Figure 3 also shows the inhibitory effects (50 to 80%) of calcium and magnesium ions on pefloxacin binding of the four strains.

EDTA susceptibility. Strains S and PT2-r were less susceptible to 1 mM EDTA (15% decrease in optical density after 20 min) than strains PT1 and PT2 were (50% decrease in optical density) (Fig. 4).

LPS and OMP banding pattern. SDS-PAGE of LPS re-

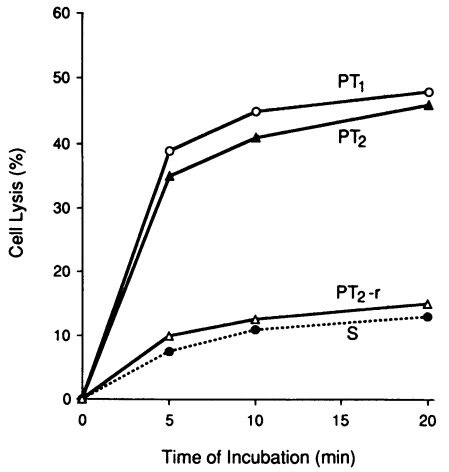

FIG. 4. EDTA susceptibility of quinolone-susceptible and quinolone-resistant *P. aeruginosa* strains. EDTA (1 mM) was added at time zero. Percent lysis was determined by comparison with controls.

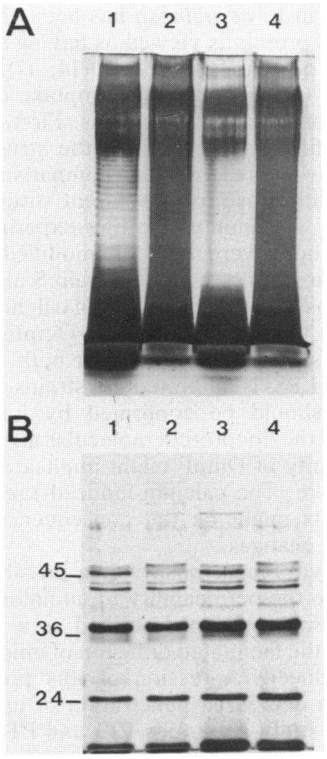

FIG. 5. SDS-polyacrylamide gel electrophoretogram of LPSs (A) and OMPs (B) of quinolone-susceptible and quinolone-resistant *P. aeruginosa* strains. (A and B) Lanes: 1, susceptible strain S; 2, posttherapy strain PT1; 3, laboratory-derived strain PT2-r; 4, posttherapy strain PT2. LPSs were prepared as described by Darveau and Hancock (13) and were separated on 14% acrylamide–4 M urea. OMPs were separated on 10% acrylamide resolving gels. The positions of migration of standard proteins are shown on the left (numbers are in kilodaltons).

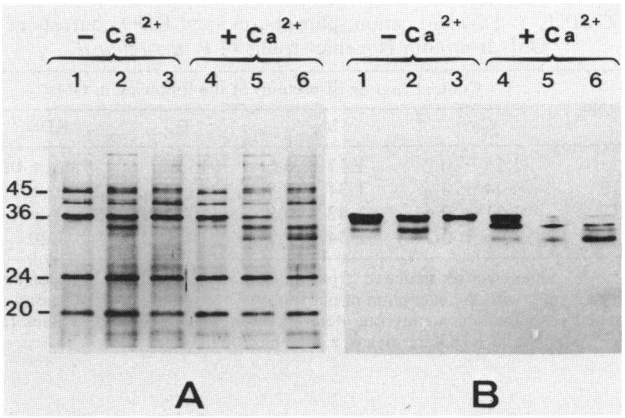

FIG. 6. Calcium-dependent electrophoretic mobility shift of OmpF of quinolone-susceptible and quinolone-resistant *P. aeruginosa* strains. (A) SDS-PAGE protein banding profile. Lanes 1 and 4, Susceptible strain S; lanes 2 and 5, posttherapy strain PT1; lanes 3 and 6, posttherapy strain PT2. In lanes 4 to 6, calcium chloride (2 mM) was added to the solubilizing mixture before heating. (B) Immunoblotting of the OMP preparations probed with anti-OmpF monoclonal antibody MA5-8. Lanes 1 to 6 are as described above for the lanes in panel A. The positions of migration of standard proteins are shown on the left (numbers are in kilodaltons).

vealed the usual heterogeneity in the sizes of the molecular elements of *P. aeruginosa* described previously (37) (Fig. 5A). Strains PT1 and PT2 were characterized by the following features when they were compared with strains S and PT2-r. (i) The ladderlike pattern corresponding to the core and O-antigen polysaccharides (33) was less visible and was somewhat masked by a uniformly stained background; (ii) the fast-moving band, which was related to the lipid A-core oligosaccharide LPS region (21) seen in the lowest part of the gel in Fig. 5A, was narrower and less stained; and (iii) on the upper part of the gel in Fig. 5A, one band with a high molecular weight was less visible.

SDS-PAGE of OMPs from the four strains (Fig. 5B) showed that they were quite similar, including expression of the major OmpF, which migrated at about 37 kDa. A narrow band that migrated in the region of D proteins, at about 47 kDa, was expressed less in strains PT1 and PT2 than it was in strains S and PT2-r. Generally, both SDS-PAGE and immunoblot preparations showed additional bands in the OmpF region migrating at 33 to 37 kDa (Fig. 6A and B, lanes 1 to 3). The 33-kDa band seemed to be the heat-nonmodifiable protein, which, particularly in strain PT1, failed to shift to the heat-modified position on the gels. When calcium chloride (2 mM) was added to the SDS-solubilized samples of OMPs before heating, the following calcium-induced alterations were observed on both SDS-PAGE and the immunoblots. (i) In strain S, the intermediate band of about 33 kDa was more pronounced (compare lanes 1 and 4 of Fig. 6B). (ii) In strains PT1 and PT2, the 37-kDa protein band tended to vanish, and an intermediate band of about 33 kDa appeared. (iii) A new low-molecular-mass band of about 31 kDa appeared in all strains. These calcium-induced electrophoretic mobility changes were concentration dependent (Fig. 7, lanes 1 to 5) and were completely reversed when the calcium-specific chelator ethyleneglycoltetraacetate (EGTA) was added (Fig. 7, lane 6). EGTA alone had no effect (Fig. 7, lane 7). The mobilities of the other bands were not altered by the presence of calcium, except that the very fast migrating band corresponding to lipoprotein I (29) on the bottom of the gel came out sharply.

KDO and ion contents of OM. OM preparations from strains PT1 and PT2 contained more Ca^{2+}, ($P < 0.05$) and more phosphorus than did those from strains S and PT2-r,

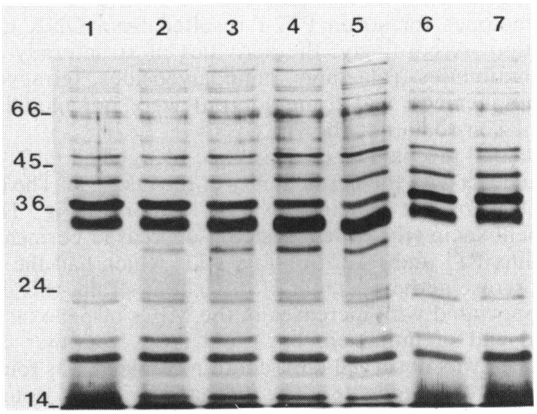

FIG. 7. Calcium concentration-dependent modification of the electrophoretic mobility of OmpF from *P. aeruginosa* PT1. Lanes: 1 to 5, 0 to 3 mM calcium; 6, 2 mM calcium with 3 mM EGTA; 7, 3 mM EGTA. The positions of migration of standard proteins are shown on the left (numbers are in kilodaltons).

TABLE 1. Divalent cation, phosphorus, and KDO contents of OMs from four isogenic strains of *P. aeruginosa*

Strain	Content (μg/mg of protein) of the following in OMs[a]:			
	Ca^{2+}	Mg^{2+}	P	KDO
S	2.25 ± 0.2	1.17 ± 0.6	15.07	7.46 ± 0.7
PT1	3.44 ± 0.3	1.34 ± 0.9	43.66	15.11 ± 1.3
PT2	3.21 ± 0.5	1.42 ± 0.9	50.14	21.31 ± 4.6
PT2-r	2.06 ± 0.2	1.34 ± 1.2	18.60	4.20

[a] All values were calculated from results of four to six independent experiments, with the exception of phosphorus assays and the KDO assay of strain PT2-r, for which only one determination was performed. Values for Ca^{2+}, Mg^{2+}, and KDO are means ± standard deviations.

while the Mg^{2+} concentration was found to be similar in the four strains. KDO concentrations were higher ($P < 0.01$) by more than 100% in strains PT1 and PT2 (Table 1). The low level of KDO in strains S and PT2-r was not due to its low level of release under hydrolysis conditions. When we doubled the time of hydrolysis, a fivefold increase in the KDO concentration was obtained with the four strains (data not shown).

DISCUSSION

In *P. aeruginosa*, alteration of DNA gyrase subunit A has been recognized as a mechanism of resistance to fluoroquinolones (19, 38), and genes associated with this resistance have been identified as *nfxA* for norfloxacin (18) and *cfxA* for ciprofloxacin (38); these are alleles of *nalA* (36). These genes have been mapped on the *P. aeruginosa* PAO chromosome between *hex-9001* and *leu-9005* (18). In this study, purification of DNA gyrase was attempted, as was done previously for the DNA gyrase of *Enterobacter cloaceae* (23), but we failed to find any DNA gyrase activity after passage in a heparin-Sepharose column, a novobiocin-Sepharose column, or both, probably because of the potent activities of the proteases produced by our strains. Indirect evidence of DNA gyrase involvement in the emergence of quinolone resistance was found in [2-^{3}H]adenine experiments with EDTA-permeabilized cells, showing that incorporation of adenine was less affected by pefloxacin in strains PT2 and PT2-r than it was in strains S and PT1. Since strains S and PT2-r had apparently similar OM permeabilities to quinolones (see below), we assumed that the 16-fold MIC increase of quinolones for strain PT2-r resulted from DNA gyrase alteration. Accordingly, the activities of structurally unrelated antibiotics (β-lactams, aminoglycosides, tetracycline, chloramphenicol, and trimethoprim) were not modified in strain PT2-r (data not shown).

A second mechanism of resistance recognized in *P. aeruginosa* deals with the permeability of the OM (31). The effect of EDTA on the labeling of [2-^{3}H]adenine (Fig. 1) and [^{14}C]pefloxacin (Fig. 3) suggested a decreased permeability in strains PT1 and PT2. In strain PT1, which had the same DNA gyrase activity as that of the parent strain, this defect was associated with increases in the MICs of pefloxacin by 16-fold and of imipenem by 4-fold (26). No other cross-resistance was observed (26), contrary to what was found in an endocarditis model (8). The mechanisms that account for this decreased permeability appear to be quite complex. Production of quinolone resistance in relation to the absence or reduced expression of OmpF has been established in *E. coli* (17) and *E. cloacae* (23). The implication that OmpF is involved in resistance remains doubtful, and even its function as a porin in *P. aeruginosa* has been challenged; in *P. aeruginosa* this protein is viewed as having a structural role similar to that of OmpA in *E. coli* (14, 47). However, the contribution of OmpF in quinolone uptake cannot be ruled out definitely for at least two reasons. First, in experiments with OmpF-deficient mutants (46), the structure of OM is probably deeply altered, making comparison of MICs for these strains with those for the parent strain difficult. Second, results of our immunoblotting experiments with the anti-OmpF antibody were markedly modified for strains PT1 and PT2 compared with those for strain S after the addition of Ca^{2+}. It is possible that this effect of calcium reflects some changes in the tertiary structure of OmpF, some specific alteration in association with LPS, or both. We have some evidence that LPS is alterated in strains PT1 and PT2, although this should be confirmed by further analyses. Modification of the lipoprotein molecular arrangement in the immediate vicinity of OmpF might implicate it indirectly in quinolone uptake. The calcium-induced rapid migration of proteins on gels might be the consequence of molecular conformational changes.

Our study favors more positively a possible contribution of protein D2 to the permeability to quinolones. Protein D2 in *P. aeruginosa* has been described as a channel that is responsible for the facilitated diffusion of imipenem (43). The absence or reduced expression of this protein has been associated with decreased penetration of imipenem (2, 24, 34, 35). In this study, in strains PT1 and PT2, we observed a reduced expression of an OMP band migrating in the region of protein D2 (apparent molecular mass, 47 kDa) and a four- to eightfold increase in the imipenem MIC. Similarly, ciprofloxacin resistance in *P. aeruginosa* clinical isolates is associated with decreased susceptibility to imipenem and reduced expression of a 43-kDa OMP band (10). Therefore, we hypothesize that the D2 porin could be a pathway for the uptake of fluoroquinolones in *P. aeruginosa*.

There is a confusing picture when one considers OMPs other than F or D2 in relation to the decreased permeability to quinolones in *P. aeruginosa*. The appearance of a 51-kDa band in a *cfxB* mutant (7) or a 54-kDa band in a *nfxB* mutant (18) and the decreased or lost expression of 25.5- or 40-kDa bands (8) indicate the complexity of this question in *P. aeruginosa* compared with the simpler pattern found in members of the family *Enterobacteriaceae*.

In addition to protein changes, with decreased levels of pefloxacin labeling, strains PT1 and PT2 were further characterized by the higher calcium and phosphorus contents of their OMs (Table 1). Ca^{2+} and Mg^{2+} have an antagonistic effect on the in vitro activity of quinolones in *P. aeruginosa* (1, 4, 5, 44). It has been hypothesized that quinolones can disorganize the OM structure (16) by chelating membrane-bound Mg^{2+} (9), further facilitating the entry of quinolones in a self-promoted pathway like the one described previously for the aminoglycosides (15). We wonder, however, whether the presence of more membrane-bound divalent cations would, by itself, diminish quinolone uptake, because the permeability-deficient strains were more and not less susceptible to EDTA, another divalent cation chelator (Fig. 4).

Higher membrane-bound calcium and phosphorus contents might reflect a higher content of LPS. Indeed, a pioneer study (39) suggested that in *Salmonella typhimurium* the branched 3-deoxy-D-mannooctulosonate trisaccharide unit of LPS may afford a specific high-affinity site for interaction with the divalent cations required for assembly and maintenance of the normal structure organization of the OM. These characteristics probably account for the fact that the OM is

enriched in both Ca^{2+} and Mg^{2+} relative to the amounts in the cytoplasmic membrane (11). Our data support this view, because (i) permeability-deficient strains contain more KDO, a molecule which is characteristic of LPS, and (ii) on SDS-PAGE of LPS, the ladderlike pattern appeared to be smeared in the permeability-deficient strains. In *P. aeruginosa*, both lipid and polysaccharide regions of LPS interact with OmpF (21), and LPS alterations have been associated with quinolone resistance (22). Many points remain unclear, however, and deserve further investigation.

In conclusion, we provided evidence that pefloxacin resistance in *P. aeruginosa* is due to (i) altered DNA gyrase activity in strain PT2-r, (ii) altered permeability in strain PT1, and (iii) both mechanisms in strain PT2. The altered permeability was associated with various alterations, among which the decreased expression of protein D2 and the higher content of calcium and LPS in the OM appear to be more directly relevant.

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