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Role of Protein D2 and Lipopolysaccharide in Diffusion of Quinolones through the Outer Membrane of *Pseudomonas aeruginosa*

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Routes of quinolone permeation in *Pseudomonas aeruginosa* were investigated by using sparfloxacin as a prototype compound. [^{14}C]sparfloxacin cell labeling was 13 to 28% lower in three protein D2-deficient mutants resistant to imipenem than in their imipenem-susceptible counterparts. In four impermeability-type quinolone-resistant strains isolated from pefloxacin-treated animals, we observed two- to fourfold-greater resistance to imipenem, reduced protein D2 expression in the outer membrane according to Western blotting (immunoblotting), and 25 to 29% decreased cell labeling with imipenem. In a protein D2-producing strain but not in its protein D2-deficient isogenic mutant, uptake of [^{14}C]sparfloxacin was strongly inhibited by L-lysine and imipenem, which act as substrates for protein D2. Conversely, binding of [^{14}C]imipenem in a porin D2-positive strain was reduced by sparfloxacin but not by the nonamphoteric quinolone nalidixic acid. Sparfloxacin, imipenem, and lysine possess a carboxyl group and a potentially protonated nitrogen separated from each other by 0.64 to 1.07 nm as calculated by computer. Hence, protein D2 may catalyze facilitated diffusion for sparfloxacin, as it does for imipenem. In addition, pefloxacin-selected isolates contained 41 to 113% more 3-deoxy-D-mannooctulosonic acid than their quinolone-susceptible counterparts, with MIC increases of 2- to 4-fold for WIN-57273 (*n*-octanol-phosphate buffer partition coefficient, 13.139), 4- to 8-fold for difloxacin (partition coefficient, 3.093) and sparfloxacin (partition coefficient, 0.431), and 8- to 16-fold for norfloxacin (partition coefficient, 0.059) and ciprofloxacin (partition coefficient, 0.056). Thus, we hypothetize that in quinolone-selected strains, increased amounts of lipopolysaccharide form a permeability barrier that acts preferentially against hydrophilic quinolones.

Pseudomonas aeruginosa has a high level of intrinsic resistance to a number of structurally unrelated antibiotics, and its outer membrane (OM) shows unusually low permeability in comparison with the membranes of most gram-negative bacteria such as *Escherichia coli* (1, 27, 44). Lower permeability in *P. aeruginosa* has been attributed to the structural characteristics of its porin channels (25). Several porin channels, including proteins C (32, 43), D1 (39), D2 (13, 37, 38, 42), E2 (42), and P (15), have been shown to allow the specific diffusion of small hydrophilic solutes. The role of protein F as a porin has been controversial (14, 41). Recently, Nikaido et al. (26) have reported that protein F acts as a major nonspecific porin for the diffusion of hydrophilic solutes but with a very low level of permeability. Protein D2 functions as a specific channel for the diffusion of lysine, arginine, ornithine, histidine, and some peptides containing these amino acids (38). It also facilitates the diffusion of imipenem and its analogs (36, 37, 41), which display affinity for the binding site of the channel. The mechanism of diffusion of hydrophobic molecules through the OM of *P. aeruginosa* has not yet been well established.

Previously, we hypothetized that protein D2 could form a pathway for the uptake of fluoroquinolones in *P. aeruginosa* (22). Several converging observations support this view. In mice infected with *P. aeruginosa* and treated with pefloxacin, analysis of posttherapy strains showed that resistance emerging during exposure to the drug was regularly accompanied by two- to fourfold-greater resistance to imipenem without alteration of other β-lactam activity (21). This observation is remarkable because pefloxacin and imipenem have quite different chemical structures that target different sites in the bacterial cell. Cross-decreased susceptibility between pefloxacin and imipenem was associated with reduced expression of an OM protein band migrating in the region of protein D2 in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (22). Quinolone-resistant *P. aeruginosa* strains emerging during exposure to ciprofloxacin (9, 30) and a spontaneous norfloxacin mutant (10) also displayed cross-resistance with imipenem, with both showing reduced expression of protein D2 (9, 10). However, routes of quinolone penetration through the *P. aeruginosa* OM are certainly multiple, and other membrane proteins are potential candidates for the mediation of diffusion (42). In addition, recent studies have suggested a possible role for lipopolysaccharide (LPS) in quinolone permeation (6–8, 19, 22).

The aim of this work was to characterize the role of protein D2 in the facilitated diffusion of sparfloxacin, which was used as a prototype quinolone compound. Both the potency of L-lysine and imipenem to impede [^{14}C]sparfloxacin labeling and the inhibition of [^{14}C]imipenem binding by sparfloxacin were studied in whole cells. We also present indirect evidence that in quinolone-resistant strains, increased amounts of LPS form a permeability barrier which acts preferentially against hydrophilic quinolones.

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TABLE 1. MICs and sources of *P. aeruginosa* strains

Strain	MIC (μg/ml)		Reference
	Imipenem	Sparfloxacin	
303 S	1	2	21
303 PT1	2	16	21
304 S	4	2	21
304 PT1	16	8	21
305 S	1	2	21
305 PT1	4	16	21
307 S	1	0.25	21
307 PT1	2	2	21
3-C	2	0.5	29
3-B	16	1	29
416	2	1	3
470	32	1	3
FRA	1	0.25	5
FRA Imi^r	8	1	5

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strains used in this study are listed in Table 1. Strains 303 S to 307 S were quinolone-susceptible isolates obtained from hospitalized patients with infections not treated by a quinolone. Strains 303 PT1 to 307 PT1 were quinolone-resistant isolates obtained from mice infected with the S counterparts and treated by a single subcutaneous dose of pefloxacin (25 mg/kg) (21). Further analysis indicated that quinolone resistance in the 305 PT1 isolate was due to altered permeability and not to DNA gyrase mutation (22). On the basis of MIC results and the cross-resistance pattern, we assume that the three other PT1 strains are also permeability-deficient mutants. Imipenem-susceptible strain 3-C and imipenem-resistant strain 3-B were isolated from the same patient (29). Strains 416 and 470 are isogenic clinical isolates, the second one showing resistance to imipenem after imipenem therapy (3). Strain FRA Imi^r was selected on imipenem-containing agar plates from strain FRA (5). Unlike their counterparts, strains 3-B, 470, and FRA Imi^r lacked expression of protein D2 (3, 5, 32, 42).

Chemicals. Antibiotics were kindly provided by the following pharmaceutical companies: imipenem, [^{14}C]imipenem (17.4 μCi/mg), and norfloxacin, Merck Sharp & Dohme Research Laboratories, Rahway, N.J.; sparfloxacin and [^{14}C] sparfloxacin (21.4 μCi/mg), Rhône-Poulenc, Paris, France, and C. E. N. Saclay, Gif-sur-Yvette, France; difloxacin, Abbott Laboratories, North Chicago, Ill.; WIN-57273, Sterling Drug Inc., Eastman Kodak Co., Rochester, N.Y.; and ciprofloxacin, Bayer AG, Leverkuusen, Germany. Other chemicals were of analytical grade.

Susceptibility testing. Susceptibility to antimicrobial agents was assayed by a microdilution method in Mueller-Hinton broth (34). The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth after 18 h at 37°C.

Partition coefficients of quinolones. The apparent partition coefficient was determined as described elsewhere (6, 13). Briefly, 1.8 ml of 0.1 M phosphate buffer (pH 7.2) was mixed with an equal volume of *n*-octanol and allowed to equilibrate for 1 h at 25°C. Then, 200 μl of the quinolone solution (100 μg/ml in 0.1 M phosphate buffer, pH 7.2) and 200 μl of *n*-octanol were added to the mixture and incubated for 24 h at 25°C with shaking. Aqueous and organic phases were separated by centrifugation for 5 min at 2,000 × *g*. The concentrations of quinolones in both phases were determined by spectrophotometry at the peak absorption for each compound. The partition coefficient was the ratio of the quinolone concentration in the organic phase to that in the aqueous phase.

Antibiotic 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 with rotatory shaking (350 rpm). Cells were harvested at 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 suspended in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (10 mM, pH 7.3) at a cell density of 16 ± 0.9 mg (wet weight) per ml (1.46 ± 0.19 mg [dry weight] per 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 with shaking, and at time zero, 50 μl of labeled antibiotic solution (final concentrations, 2.5 μg/ml for [^{14}C]sparfloxacin and 10 μg/ml for [^{14}C]imipenem) was added. After 5 min of incubation, 50 μl of nonradioactive imipenem or sparfloxacin (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 had been prewetted with the same unlabeled antibiotic solution. Filters were rapidly washed three times 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 antibiotic that bound to filters without bacteria was measured and subtracted from the results. Experiments were run in parallel for each pair of strains.

Inhibition assays. The effects of L-lysine (final concentration, 0.1 to 100 mM) and imipenem (final concentration, 0.1 to 2.5 mM) on [^{14}C]sparfloxacin labeling were determined with strains 305 S and 305 PT1. Conversely, inhibition of [^{14}C]imipenem uptake by quinolones and L-lysine was determined for strain 3-C and 3-B. Decreased binding of both labels was calculated as a percentage of the values obtained when no inhibitor was added. Incubation time of cells with the inhibitors was 5 min.

OM preparation. Cell envelopes and OMs were prepared as described by Spratt (33) and Marchou et al. (20). 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] per 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, suspended in distilled water at a protein concentration of about 10 mg/ml, and stored at −20°C. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.). Bovine serum albumin was used as a standard.

Gel electrophoresis and immunoblotting. OM proteins were analyzed by SDS-PAGE with a Protean II Slab Electrophoresis Cell (Bio-Rad Laboratories, Richmond, Calif.). OM protein fractions were solubilized in sample buffer, heated at 95°C for 5 min, and electrophoresed on a 10% acrylamide resolving gel at a constant current of 10 mA per gel.

TABLE 2. Apparent partition coefficients in *n*-octanol and fold increases in MICs of five quinolones against *P. aeruginosa* strains

Quinolone	Partition coefficient	Fold increase in MIC for strain:						
		303 PT1	304 PT1	305 PT1	307 PT1	470	3-B	FRA Imi^r
Ciprofloxacin	0.056	8	16	16	8	1	2	4
Norfloxacin	0.059	16	16	16	8	1	2	4
Sparfloxacin	0.431	8	4	8	8	1	2	4
Difloxacin	3.099	5	4	4	4	1	2	2
WIN-57273	13.139	3	4	2	2	1	2	4

Subsequently, gels were silver stained after treatment with glutaraldehyde (28).

For protein D2 immunoblotting, OM proteins 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 elsewhere (35) by using rabbit anti-*P. aeruginosa* protein D2 antibody. Bound antibody was detected with a goat anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands) by using 4-chloro-1-naphthol and hydrogen peroxide as reagents.

KDO assay. Levels of 3-deoxy-D-mannooctulosonic acid (KDO) were determined by the colorimetric assay of Karkhanis et al. (18) with OM samples hydrolyzed in 0.2 N H_2SO_4 for 25 min at 100°C. The ammonium salt of KDO was used as a standard.

Molecular structure analysis. Interatomic distances in the molecules of lysine, imipenem, and sparfloxacin were calculated by the MOLOC program (Hoffmann-La Roche Ltd., Basel, Switzerland), which is a computer modeling system providing databases in pharmaceutical research (24).

RESULTS

Antibiotic susceptibility and partition coefficients of quinolones. Compared with their pretherapy counterparts, all PT1 strains showed a 2- to 16-fold increase in MICs of the five quinolones tested (Table 2) and a 2- to 4-fold increase in MICs of imipenem (Table 1). Among imipenem-resistant strains which were not exposed to quinolones, strains 3-B and FRA Imi^r exhibited a 2- to 4-fold increase in MICs of quinolones compared with the initial strains (Table 2). Quinolone activity remained unchanged in strain 470 (Table 2). The selected quinolones represented a large pattern of octanol-phosphate buffer partition coefficients that varied from 0.056 to 13.14 (Table 2). After exposure of strains to pefloxacin, we observed 8- to 16-fold increases in MICs of ciprofloxacin and norfloxacin, which are the most hydrophilic molecules, and only 2- to 4-fold increases in MICs of WIN-52273, which is the most hydrophobic molecule. Sparfloxacin and difloxacin showed intermediate results with respect to both partition coefficients and MIC increases. In contrast, alteration of quinolone activities was not dependent on partition coefficients in strains 3-B and FRA Imi^r.

KDO assays. KDO concentrations were significantly higher (41 to 113%) in the PT1 strains than in the corresponding S isolates (Table 3). By contrast, KDO contents of preparations from the strain pairs not exposed to quinolones remained unchanged.

OM banding pattern. Strains 3-C and 3-B were used as controls for the presence and absence of protein D2 (37) (Fig. 1A and B, respectively). SDS-PAGE of OM proteins from strains 416 and 470 revealed similar patterns for proteins F, G, H, and I, as previously reported by Bellido et al. (3). The protein band migrating at about 45 kDa in strain 416 and lacking expression in strain 470 (Fig. 1A, lane 4) showed D2 antigenicity in strain 416 (Fig. 1B, lane 4).

Strains 303 PT1 and 304 PT1 but not 305 PT1 and 307 PT1 clearly showed decreased expression of a narrow band migrating at 47 kDa, i.e., in the region of protein D2 (Fig. 2A). A similar finding was previously reported for 305 PT1 (22). After being immunoblotted with anti-protein D2 antibody, the expression of porin D2 diminished in all posttherapy isolates (Fig. 2B).

Radiolabeling of whole cells. To compare the labeling of whole cells by [^{14}C]imipenem and [^{14}C]sparfloxacin at steady state, the amounts of radioactivity retained by the bacteria were measured after 5 min of incubation with the antibiotic in five pairs of strains (Table 4). Considerable variations in the amount of cell-bound antibiotic in pretherapy isolates were found: 40 to 158 ng/mg (dry weight) of bacteria with [^{14}C]imipenem and 52 to 207 ng/mg (dry weight) with [^{14}C]sparfloxacin. In all cases, less radioactivity was counted in resistant isolates than in susceptible isolates, with the decrease varying from 25 to 59% in [^{14}C]imipenem assays and from 13 to 52% in [^{14}C]sparfloxacin assays.

Inhibition assays. Figure 3 shows the time course of sparfloxacin uptake in strains 305 S and 305 PT1. In the presence of L-lysine, binding was inhibited by 40% in strain 305 S. A 50% inhibitory concentration of 7 mM was calculated from the data in Fig. 4. We were not able to determine the 50% inhibitory concentration for imipenem because of limited solubility of the drug. However, imipenem appeared

TABLE 3. KDO assays of OM protein

Strain	No. of determinations	KDO concn ± SD (μg/mg of OM protein)	% Variation (post- vs pretherapy)	*P* value (*t* test)[a]
303 S	6	59.76 ± 23		
303 PT1	6	94.34 ± 60	+57.8	<0.05
304 S	7	30.52 ± 10		
304 PT1	7	65.12 ± 19	+113.4	<0.01
305 S	4	36.51 ± 4		
305 PT1	4	64.32 ± 16	+76.2	<0.05
307 S	3	39.03 ± 4		
307 PT1	3	55.22 ± 13	+41.5	<0.05
416	9	38.70 ± 6		
470	9	37.34 ± 13	−3.5	NS
3-C	9	53.37 ± 22		
3-B	9	58.04 ± 19	+8.7	NS
FRA	6	95.73 ± 27		
FRA Imi^r	6	82.03 ± 17	−14.3	NS

[a] NS, not significant.

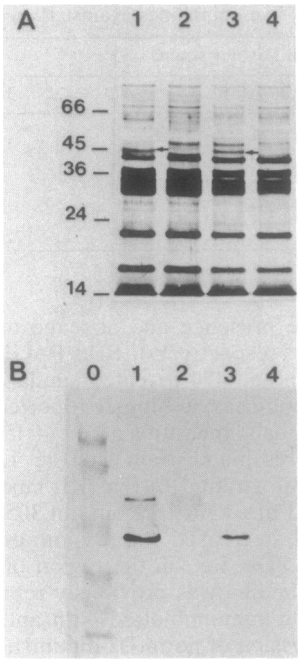

FIG. 1. (A) SDS-polyacrylamide gel electrophoretogram of OM proteins of imipenem-susceptible and imipenem-resistant *P. aeruginosa* strains. OM proteins were separated on 10% acrylamide resolving gels and were silver stained. The migration positions of standard proteins are shown on the left (in kilodaltons). Levels of protein D2 migration are indicated by the arrows. (B) Immunoblotting of OM protein preparations probed with anti-protein D2 antibody. Lane 0, prestained standard proteins. (A and B) Lanes: 1, strain 3-C; 2, strain 3-B; 3, strain 416; 4, strain 470.

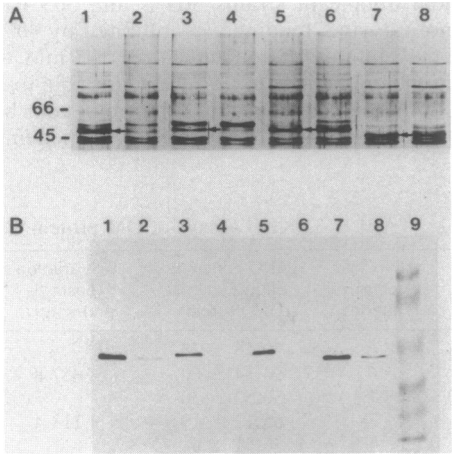

FIG. 2. (A) SDS-polyacrylamide gel electrophoretogram of OM proteins of quinolone-susceptible and quinolone-resistant *P. aeruginosa* strains. OM proteins were separated on 10% acrylamide resolving gels and were silver stained. Levels of protein D2 migration are indicated by the arrows. The migration positions of standard proteins are shown on the left (in kilodaltons). (B) Immunoblotting of the OM protein preparations probed with anti-protein D2 antibody. Lane 9 shows prestained standard proteins. (A and B) Lanes: 1, strain 303 S; 2, 303 PT1; 3, 304 S; 4, 304 PT1; 5, 305 S; 6, 305 PT1; 7, 307 S; 8, 307 PT1.

TABLE 4. Whole-cell labeling of *P. aeruginosa*

^{14}C-labeled antibiotic	Strain pairs	Cell labeling[a]		
		Pretherapy (ng/mg [dry wt] of cells)	Posttherapy (ng/mg [dry wt] of cells)	% Decrease[b]
Imipenem	303 S/303 PT1	40.1 ± 8 (3)	28.8 ± 6 (3)	29
	305 S/305 PT1	41.9 ± 8 (5)	31.4 ± 5 (3)	25
	3-C/3-B	62.1 ± 3 (3)	25.2 ± 4 (3)	59
	416/470	120.7 (1)	66.9 (1)	45
	FRA/FRA Imi^r	158.7 ± 15 (3)	99.3 ± 26 (3)	37
Sparfloxacin	303 S/303 PT1	52.5 ± 21 (5)	25.2 ± 3 (6)	52
	305 S/305 PT1	83.9 ± 44 (4)	58.6 ± 40 (4)	30
	3-C/3-B	108.3 ± 17 (5)	78.9 ± 12 (6)	27
	416/470	79.4 ± 14 (4)	68.7 ± 8 (3)	13
	FRA/FRA Imi^r	207.6 ± 72 (3)	148.0 ± 43 (3)	28

[a] Mean ± standard deviation after 5 min of incubation with [^{14}C]imipenem and [^{14}C]sparfloxacin. Numbers in parentheses are numbers of determinations.
[b] Posttherapy compared with pretherapy.

to be a more active inhibitor than L-lysine when compared on a molar basis within the range of concentrations tested. L-Lysine and imipenem had no inhibitory effects on sparfloxacin labeling of strain 305 PT1 (data not shown). Imipenem binding in strain 3C was inhibited by both 5 mM L-lysine and 5 mM sparfloxacin (Fig. 5), while nalidixic acid and WIN-57273 at the same molar concentrations had no significant effect on labeling. Quinolones and L-lysine did not alter imipenem uptake in control experiments performed with strain 3-B (Fig. 5).

Structure analysis. As shown in Fig. 6, interatomic distances between positive (amino-group) and negative (carboxyl-group) charges, which were separated from each other by 6 to 8 bond lengths, were calculated to be 0.65 to 0.76 nm for L-lysine (depending on the crystallized form) and 0.79 nm for imipenem. In the sparfloxacin molecule, the distance

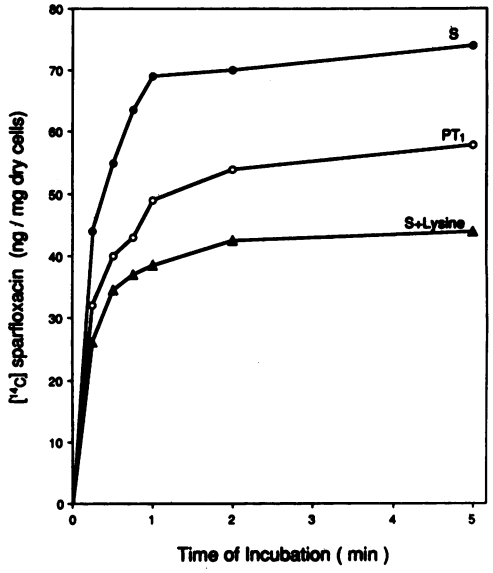

FIG. 3. Time-course of [^{14}C]sparfloxacin cell labeling in quinolone-susceptible 305 S and quinolone-resistant 305 PT1 strains (○) of *P. aeruginosa*. Labeling in strain 305 S was determined in the presence (▲) and the absence (●) of 1 mM L-lysine.

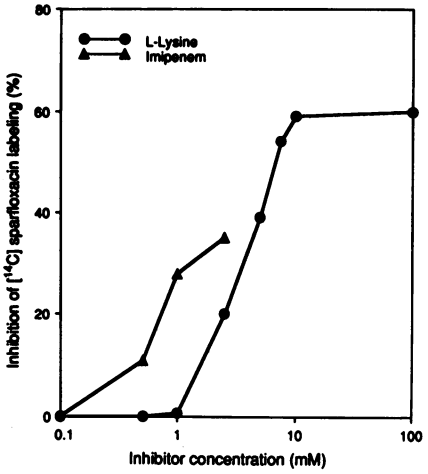

FIG. 4. Inhibition of [^{14}C]sparfloxacin whole-cell labeling by L-lysine and imipenem in quinolone-susceptible *P. aeruginosa* 305 S. Standard conditions were used as described in the text.

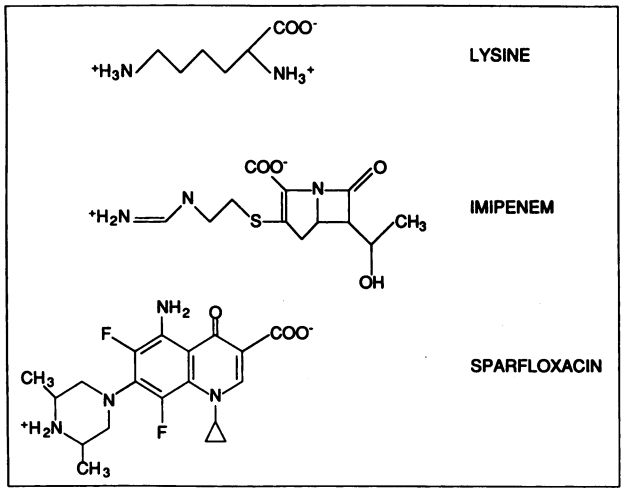

FIG. 6. Structures of lysine, imipenem, and sparfloxacin. Positive and negative charges are indicated.

between the carboxyl group and the potentially protonated nitrogen at position 4 of the piperazine moiety was found at 1.07 nm (Fig. 6).

DISCUSSION

The first lines of evidence indicating a possible role for protein D2 in quinolone permeation were provided by the studies showing that cross-resistance with imipenem in quinolone-resistant *P. aeruginosa* of the impermeability type (9, 10, 21, 30) was associated with reduced expression of protein D2 (9, 10, 22). This was confirmed here, but we also found two of three imipenem-resistant, protein D2-deficient strains displaying cross-resistance with quinolones. Strain 470 is distinctive in combining porin D2 deficiency (contributing to a fourfold increase in the MIC of imipenem) with reduced affinity of PBP4 for imipenem (accounting for an additional fourfold MIC increase) (3). The rather limited permeability reduction in this case may account for the small decrease in [^{14}C]sparfloxacin binding and the lack of MIC changes with quinolones.

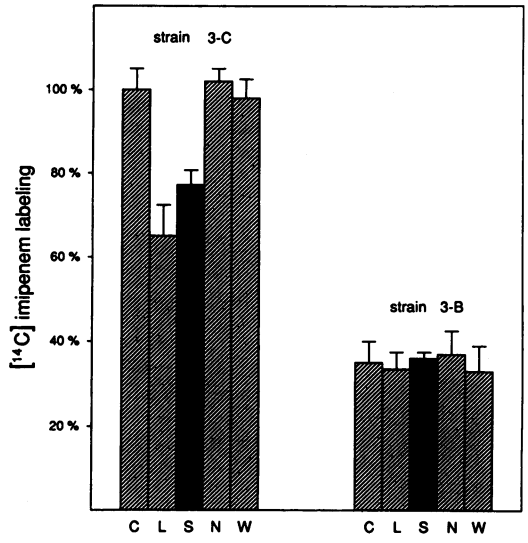

FIG. 5. Effects of 0.5 mM L-lysine (L), sparfloxacin (S), nalidixic acid (N), and WIN-57273 (W) on imipenem uptake in *P. aeruginosa* 3-C and 3-B. Labeling was determined after 10 s of [^{14}C]imipenem (5 μg/ml) incubation. Uptake values were related to 100% for the control (C). Standard deviations are indicated for each bar.

Further interesting analogies in the resistant strains under study were observed. Whatever the selective agent leading to resistance, protein D2 was under- or unexpressed and [^{14}C]imipenem and [^{14}C]sparfloxacin cell labelings were reduced.

Inhibition assays produced a second line of evidence. L-Lysine and imipenem, both of which are substrates for the protein D2 channel, appeared to be strong inhibitors of [^{14}C]sparfloxacin labeling in strain 305 S but not in protein D2-deficient strain 305 PT1. Conversely, L-lysine and sparfloxacin both competed with [^{14}C]imipenem in a binding assay performed with protein D2-positive strain 3-C. These results indicate that sparfloxacin has affinity for the protein D2-binding site. Thus, porin D2 may catalyze facilitated diffusion of the quinolone, as it does for imipenem. Interestingly we found imipenem to be a stronger inhibitor of [^{14}C] sparfloxacin binding than the basic amino acid L-lysine. In accordance with this finding, channel affinity has been shown to be lower toward the basic amino acids than toward the unnatural substrate imipenem (38).

To explain the affinity for protein D2, the presence, location, and role of the carboxyl group combined with a positively charged group in both basic amino acids and imipenem have already been discussed (38). Similarly, sparfloxacin behaves as an amphotere containing an anionic carboxyl group combined with a protonable nitrogen in the piperazine moiety (Fig. 6). With great interest, we found that the interatomic distances between the oppositely charged groups in L-lysine, imipenem, and sparfloxacin were quite comparable. Hence, it seems likely that sparfloxacin, like imipenem (38), mimics the structure of natural substrates for porin D2, notably basic amino acids and peptides.

However, inhibition of [^{14}C]sparfloxacin labeling by L-lysine was incomplete, achieving a maximal value of only 60%. This suggests that for crossing the OM, sparfloxacin can use alternative pathways not available to L-lysine.

Several proteins are candidates for this pathway, notably porins E2 (42) and G (6) as well as LPS (6, 8).

LPS changes in pefloxacin-selected strains raise the possibility that the reduced presence of protein D2 is a secondary phenomenon. Recent studies have documented the close relationship between LPS and PhoE porin (17). Accordingly, increased amounts of LPS in the OM could impede protein D2 anchorage. However, imipenem-selected strain 3-B displayed a clearly diminished amount of D2 protein without changes in the KDO content, suggesting a role for D2 in the quinolone uptake independent of LPS changes. Furthermore, the increase in KDO content was associated with hydrophilicity-dependent MIC increases. Increased LPS content in *P. aeruginosa* could produce a permeability barrier restricting penetration of the hydrophilic quinolones (ciprofloxacin, norfloxacin), as it does for other hydrophilic antibiotics such as beta-lactams (11, 12) and aminoglycosides (4). Along the same lines, a liposome-binding assay showed that hydrophobic forces were involved in the binding of quinolones to phospholipids (2). In apparent contradiction, however, a study with a rough mutant of *Salmonella typhimurium* (16) suggested that the LPS layer forms a permeability barrier for hydrophobic but not for hydrophilic quinolones.

In conclusion, we showed that the position of opposite electric charges and hydrophobicity were important for quinolone permeation through the OM in *P. aeruginosa*. These molecular characteristics are not necessarily those leading to affinity for the target site. Better knowledge of the parameters could yield a new drug design for quinolones aimed specifically at improved permeation, an issue especially important with *P. aeruginosa*, in which resistance to quinolones is indeed critical (23, 31, 40).

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