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Routes of Quinolone Permeation in *Escherichia coli*

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The uptake of quinolone antibiotics by *Escherichia coli* was investigated by using fleroxacin (RO 23-6240, AM 833) as a prototype compound. The uptake of fleroxacin was reduced and its MIC was increased in the presence of magnesium. Quinolones induced lipopolysaccharide release, increased cell-surface hydrophobicity and outer membrane permeability to β-lactams, and sensitized cells to lysis by detergents. These effects were also antagonized by magnesium and were very similar to those seen with EDTA and gentamicin. MICs of quinolones in porin-deficient strains were increased relative to those of the parent strain, consistent with a porin pathway of entry. However, MICs were further increased in the presence of magnesium; the size of the additional increase showed a positive correlation with quinolone hydrophobicity in an OmpF$^-$ OmpC$^-$ OmpA$^-$ strain. When quinolones were mixed with divalent cations in solution, changes in quinolone fluorescence suggestive of metal chelation were observed. The addition of fleroxacin to a cell suspension resulted in a rapid initial association of fluorescence with cells, followed by a brief decrease and a final time-dependent linear increase in cell-associated fluorescence. We interpret these results as representing chelation of outer membrane-bound magnesium by fleroxacin and other quinolones, dissociation of the quinolone-magnesium complex from the outer membrane, and diffusion of the quinolone through both porins and exposed lipid domains on the outer membrane. For a given quinolone, the contribution of the porin and nonporin pathways to total uptake is influenced by the hydrophobicity of the quinolone.

Quinolones are broad-spectrum antibacterial agents whose primary mechanism of action is inhibition of DNA gyrase activity (2). Access to the target site is a major determinant of antibacterial activity, the outer membrane being the major permeability barrier in gram-negative bacteria. Quinolones are known to penetrate the outer membrane of *Escherichia coli* through the OmpF and OmpC porins; this has been demonstrated by both the use of defined porin-deficient strains (8) and the isolation of quinolone-resistant mutants that lack porins (9, 10). However, the size of the quinolone MIC increase in porin-deficient mutants relative to the wild type is rarely more than fourfold. In similar porin-deficient mutants, the MIC increases of hydrophilic cephalosporins, which are restricted to the use of porins (18, 25), may be as much as 64-fold (11). Hirai et al. (8) noted that the MIC of quinolones was decreased in lipopolysaccharide-deficient (rough) mutants as compared with the wild type, and the size of the decrease correlated with the hydrophobicity of the quinolone. These observations suggested that the passage of quinolones through the outer membrane was not limited to porins.

Several classes of antibiotics, including the aminoglycosides and polymyxin B, are known to penetrate the outer membrane by pathways other than porins (6). These molecules displace divalent cations which bridge adjacent lipopolysaccharide molecules, thus destabilizing the outer membrane and exposing areas of the lipid bilayer (15, 21) through which antibiotics can then diffuse. The uptake of these polycationic antibiotics has been termed "self-promoted" (6).

In the present study, the interaction of fleroxacin with the outer membrane of *E. coli* was examined in an effort to determine its mechanism of uptake.

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MATERIALS AND METHODS

Materials. Fleroxacin (RO 23-6240, AM 833) and polymyxin B nonapeptide were obtained from Hoffmann-La Roche, Inc. (Nutley, N.J.). Norfloxacin was from Merck & Co., Inc. (Rahway, N.J.); ofloxacin was from Ortho Diagnostics, Inc. (Raritan, N.J.); pefloxacin was from Rhone-Poulenc Pharmaceuticals (Monmouth Junction, N.J.); difloxacin (A-56619) and A-56620 were from Abbott Laboratories (North Chicago, Ill.); amifloxacin was from Sterling-Winthrop Research Institute (Rensselaer, N.Y.); CI-934 was from Warner-Lambert (Ann Arbor, Mich.); nalidixic acid, oxolinic acid, pipemidic acid, gentamicin, EDTA, glycine, and 2,4-dinitrophenol were from Sigma Chemical Co. (St. Louis, Mo.); ampicillin was from Wyeth Laboratories (Philadelphia, Pa.), and penicillin G was from Eli Lilly & Co. (Indianapolis, Ind.). L-[U-^{14}C]proline (specific activity, 250 mCi/mmol) was from New England Nuclear Corp. (Boston, Mass.).

Strains and culture conditions. The strains used in this work are listed in Table 1. Strains were routinely grown in Luria broth at 37°C in a shaking water bath.

Bacterial growth. The growth of bacterial cultures in the presence or absence of various compounds was determined by monitoring the optical density of the culture at 600 nm. Cultures of 25 ml were grown in 125-ml sidearm flasks at 37°C with shaking, and the optical density of the culture was determined at 20-min intervals in a Bausch & Lomb Spectronic 20.

MIC determination. MICs were determined by broth dilution in 48-well microdilution plates by using a volume of 1.0 ml and an inoculum of 5 μl of an early-log-phase culture (~5 × 10^4 cells per ml). Plates were incubated for 18 h at 37°C, and the MIC was determined visually. The concentration of $MgCl_2$ in magnesium-supplemented broth was 5.0 mM.

Quinolone uptake. The uptake of fleroxacin was monitored by a fluorometric method (J. S. Chapman and N. H. Geo-

TABLE 1. *E. coli* strains

Strain	Characteristics	Source
JF568	K-12 *aroA57 cyc-1 his-53 ilv-277 lacY29 metB65 purE41 rpsL77 tsx-63 xyl-114*	J. Foulds (18)
JF694	JF568 *ompC624 ompF254 nmpA1* (OmpC$^-$ OmpF$^-$ PhoE$^+$)	J. Foulds
JF701	JF568 *ompC624* (OmpC$^-$)	J. Foulds
JF703	JF568 *ompF254* (OmpF$^-$)	J. Foulds
CS1293	K-12 *ompC178 ompF168 ompA159* (OmpC$^-$ OmpF$^-$)	C. Schnaitman
MAL300	*ecfA*(Ts) *metC strA thi*	*E. coli* Genetic Stock Center (14)
EC1005	*gyrA* (Nal^r) *metA*	D. Clark (3)
RC709	K-12 R1$^+$ (Amp^r) *met-2 pro-1 thi*	Roche Culture Collection (17)
25922	Clinical isolate	American Type Culture Collection

rgopapadakou, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 623, 1987). Cells were grown to an A_{660} of 0.4, harvested by centrifugation, washed once with 50 mM sodium phosphate buffer (pH 7.2), and suspended in the same buffer to 20 A_{660} units per ml (approximately 40 mg [wet weight] per ml). Fleroxacin was added to a final concentration of 10 μg/ml, and at appropriate time intervals 0.5-ml samples were removed and diluted into 2.0 ml of phosphate buffer. The cells were pelleted (5,600 × *g* for 1 min), washed once with 2.0 ml of buffer, and treated with 2.0 ml of 0.1 M glycine hydrochloride (pH 3.0) for 1 h. The samples were centrifuged (5,600 × *g* for 5 min), and the fluorescence of the supernatant was determined at 442 nm with excitation at 282 nm.

In some experiments, cells were treated with 2.0 mM dinitrophenol for 10 min before the addition of fleroxacin. In uptake experiments involving the temperature-sensitive strain MAL300, the cells were incubated at either 37 or 43°C for 15 min before the addition of fleroxacin. In some experiments, [^{14}C]proline was substituted for fleroxacin, and the samples were processed as described above for fleroxacin uptake. Cell-associated radioactivity was determined by liquid scintillation counting.

Quinolone hydrophobicity. The hydrophobicity of the quinolones was determined by partition between 0.1 M phosphate buffer (pH 7.2) and *n*-octanol as described by Hirai et al. (8), with the following modifications. The buffer-octanol mixture was incubated for 24 h instead of 48 h, and both the buffer and the *n*-octanol were equilibrated with each other before the addition of the quinolone to eliminate the observed volume changes of the two phases. The partition coefficient (*P*) is expressed as the ratio of the quinolone concentration in the organic phase to that in the aqueous phase. Quinolone concentrations were determined by UV absorbance at 280 nm.

Cell-surface hydrophobicity. Hydrophobicity was determined by a modification of the method of Hirai et al. (9). Bacteria were grown in Luria broth to an A_{660} of 0.2, harvested by centrifugation, washed once with 50 mM sodium phosphate (pH 7.2), and suspended to an A_{660} of 1.0. Cells were incubated with 100 μg of fleroxacin or gentamicin per ml or with 5.0 mM EDTA for 5 min, washed twice, and suspended to their original volume in 50 mM sodium phosphate buffer (pH 7.2). A 2.5-ml sample of bacterial suspension was mixed with 1.0 ml of *p*-xylene by vortexing for 2 min and then allowed to separate at room temperature for 15 min. The cell-surface hydrophobicity (P_s) was calculated as (A_{660} of untreated control − A_{660} of treated sample)/A_{660} of untreated control.

Outer-membrane permeability. The permeability of the outer membrane to penicillin G was measured by determining the rate of its hydrolysis by intact cells of *E. coli* RC709. Cells were grown in Luria broth to an A_{660} of 0.4, harvested, suspended in 50 mM sodium phosphate buffer (pH 7.0) to 1 A_{660} unit per ml, and exposed to the test compound for 60 min. Cells were washed once to remove the test compound and any β-lactamase in the supernatant. After cells were exposed to the test compound, the β-lactamase activity of the cell suspension was determined after 10 min of incubation with 1.0 mM penicillin G and corrected for any β-lactamase activity remaining in the supernatant. Hydrolysis of penicillin G was determined by the iodometric method of Sargent (20).

RESULTS

Uptake of fleroxacin. Consistent with uptake being a diffusive process (D. C. Hooper, J. S. Wolfson, K. S. Souza, and M. N. Swartz, 26th ICAAC, abstr. no. 944, 1986) (1), uptake of fleroxacin by *E. coli* JF568 was nonsaturable from 0.01 to 100 μg of fleroxacin per ml (0.027 to 270 μM). A time course of fleroxacin uptake in the absence and presence of $MgCl_2$ is shown in Fig. 1. After the addition of fleroxacin to the cell suspension, there was a rapid association of fleroxacin with the cells, followed by a decrease in association after 15 to 20 min and then a linear increase in cell-associated fleroxacin. These complex kinetics were identical in dinitrophenol-poisoned cells and in cells suspended in buffer with and without glucose. A further demonstration that maintenance of proton motive force via respiration or ATP hydrolysis is not necessary for quinolone uptake is provided by transport experiments with *E. coli* MAL300. This strain is unable to maintain a transmembrane potential at elevated temperatures (13) and presents identical kinetics of fleroxacin association with cells at both permissive and restrictive temperatures. Control experiments with [^{14}C]proline confirmed the presence or absence of active transport under these conditions. Fleroxacin uptake experiments performed with *E. coli* ATCC 25922 (data not shown) showed the same type of kinetics as with *E. coli* JF568 and *E. coli* MAL300, confirming that the kinetics are not strain specific.

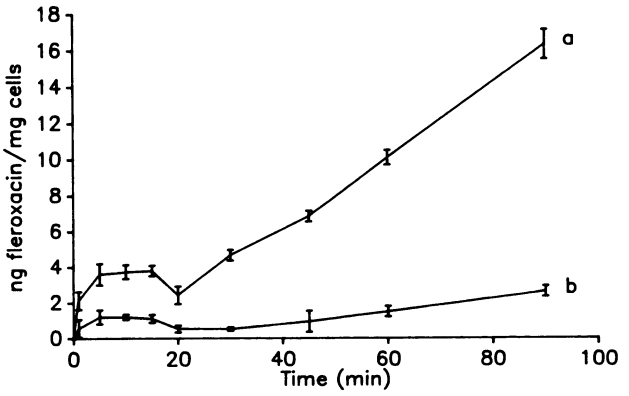

FIG. 1. Uptake of 10 μg of fleroxacin per ml by *E. coli* JF568 without (a) and with (b) added 10.0 mM $MgCl_2$.

TABLE 2. MICs of fleroxacin for *E. coli* strains

Strain	MIC (μg/ml)	
	Without $MgCl_2$	With $MgCl_2$[a]
JF568	0.1	0.3
JF694	0.25	ND[b]
JF701	0.1	ND
JF703	0.45	0.8
CS1293	0.25	0.6
EC1005	1.0	2.0

[a] Concentration of $MgCl_2$ was 5.0 mM.
[b] ND, Not determined.

The prior addition of 10 mM $MgCl_2$ to the cell suspension reduced the amount of cell-associated fleroxacin in the first phase by 69%. The rate at which fleroxacin was lost by cells 15 to 20 min after substrate addition was similar in buffer with or without $MgCl_2$ (6.2 ± 2.4 and 7.2 + 0.8%/min, respectively). The rate of uptake in the third, linear phase was reduced from 0.194 to 0.035 ng of fleroxacin per mg of cells per min, a drop of over 80%.

Effect of cations on MICs. The reduced uptake of fleroxacin in the presence of $MgCl_2$ was reflected in increased MICs of fleroxacin (Table 2). The antagonism between fleroxacin and magnesium occurred in wild-type, porin-deficient, and gyrase-resistant strains, and the magnitude of the increase was similar to that seen between porin-deficient mutants and their parent strain (Table 2). Increasing concentrations of magnesium correlated with increasing MICs at least up to 100 mM $MgCl_2$; at this concentration a white precipitate formed with more than 5 to 10 μg of fleroxacin per ml. Manganese and calcium also antagonized the growth inhibition by fleroxacin, whereas sodium and potassium were ineffective.

MICs determined for a number of quinolones with and without 5 mM $MgCl_2$ with an $OmpF^-$ $OmpC^-$ $OmpA^-$ strain, CS1293, revealed a relationship between MIC increases and quinolone hydrophobicity (Table 3). There is a positive correlation of 0.727 between increasing hydrophobicity of all the compounds in Table 3 and the size of their MIC increase in the presence of magnesium. Limiting the data set to fluoroquinolones bearing piperazine rings increases the coefficient of correlation to 0.987.

Effect of cations on quinolone fluorescence. At pH 7.5, magnesium and calcium increased the fluorescence of fleroxacin at 442 nm to 175 and 110% of the control, whereas manganese decreased the fluorescence to 22% of the control (Fig. 2). The fluorescence of norfloxacin and pefloxacin (50 ng/ml) in the presence of magnesium (5 mM) increased to 192 and 174%, respectively, of that of controls.

TABLE 3. Ratio of MICs of quinolones determined in the presence and absence of 5 mM $MgCl_2$ with *E. coli* CS1293

Compound	MIC ratio	P_{app}[a]
A-56620	1.5	0.085
Ciprofloxacin	2	0.151
Fleroxacin	2.3	0.140
Nalidixic acid	2.5	0.531
Norfloxacin	2.5	0.162
Ofloxacin	2.7	0.196
Pefloxacin	4.25	0.529
Pipemidic acid	5.0	0.150
CI-934	5.0	0.168
Amifloxacin	6.0	0.602
Difloxacin (A-56619)	6.2	0.735
Oxolinic acid	7.5	0.742

[a] P_{app}, Apparent hydrophobicity index. An increase in P_{app} reflects an increase in hydrophobicity.

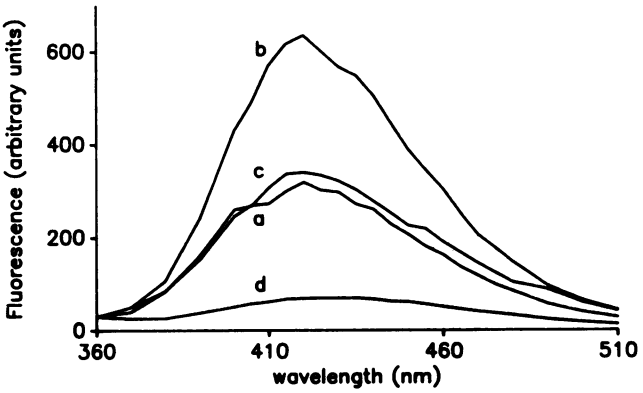

FIG. 2. Fluorescence spectra of a fleroxacin solution (50 ng/ml) (excitation wavelength, 282 nm): a, no additions; b, 5.0 mM $MgCl_2$; c, 5.0 mM $CaCl_2$; d, 5.0 mM $MnCl_2$.

Potassium and sodium had no effect on the fluorescence of 50 ng of fleroxacin per ml even when present at 5 mM. Changes in the fluorescence of fleroxacin were seen with magnesium/fleroxacin molar ratios as low as 0.1. The fluorescence changes were concentration dependent and displayed saturation at higher magnesium concentrations (data not shown). The largest changes in fluorescence, with fixed concentrations of fleroxacin and magnesium, were seen between pH 7 and 8.

Quinolone effects on the outer membrane. The rate of hydrolysis of penicillin G by *E. coli* RC709 cells treated with fleroxacin was 120% of the rate of untreated cells (Table 4). Magnesium prevented the increase in the rate of hydrolysis by quinolone-treated cells and in fact reduced the rate by 60%. Treatment with nalidixic acid produced a smaller increase in the rate of hydrolysis, as did treatment with streptomycin. EDTA produced the largest increase in the hydrolytic rate.

At 5.0 μg/ml, fleroxacin sensitized cells of the normally resistant JF568 to lysis by sodium dodecyl sulfate (SDS) (Fig. 3). A similar effect was seen with fleroxacin at 2.5 μg/ml but not at 1.0 μg/ml. Gentamicin, a polycation, and EDTA, a chelator, also sensitized *E. coli* JF568 to lysis by SDS (Fig. 3). *E. coli* ATCC 25922, a clinical isolate, and *E. coli* EC1005, a quinolone-resistant strain, were also sensitized to lysis by SDS (data not shown). Magnesium prevented sensitization to SDS lysis by fleroxacin, EDTA, and gentamicin but did not fully alleviate growth inhibition by fleroxacin.

Direct measurements of the cell-surface hydrophobicity by partitioning of cells between buffer and *p*-xylene revealed

TABLE 4. Hydrolysis of penicillin G by *E. coli* RC709 after different treatments

Compound added	Relative hydrolytic rate
None	100
Fleroxacin (10 μg/ml)	120.9 ± 5.0
Fleroxacin (10 μg/ml) + $MgCl_2$ (10 mM)	40.4 ± 8.1
Nalidixic acid (20 μg/ml)	106.2 ± 1.7
Streptomycin (10 μg/ml)	112.2 ± 6.8
EDTA (5 mM)	133.2 ± 5.9

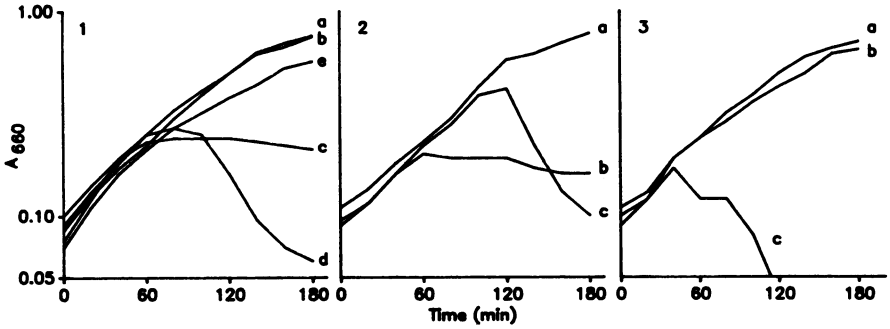

FIG. 3. Effect of fleroxacin, gentamicin, and EDTA on SDS-induced lysis of *E. coli* JF568. Cells were grown in Luria broth, and indicated additions were made 40 min after inoculation. Panel 1: a, no additions; b, 0.1% SDS; c, 5.0 μg of fleroxacin per ml; d, fleroxacin and 0.1% SDS; e, fleroxacin, SDS, and 10 mM $MgCl_2$. Panel 2: b, 20 μg of gentamicin per ml; c, gentamicin and SDS; a, gentamicin, SDS, and $MgCl_2$. Panel 3: a, 5.0 mM EDTA; c, EDTA and SDS; b, EDTA, SDS, and $MgCl_2$.

that treatment of cells with fleroxacin increased the cell-surface hydrophobicity by 35% (Table 5). Gentamicin and EDTA caused similar increases in cell-surface hydrophobicity, whereas the polycation polymyxin B nonapeptide caused an increase of 71%. Magnesium prevented the increase in cell-surface hydrophobicity caused by fleroxacin and in fact reduced the cell-surface hydrophobicity compared with that of the control. Ampicillin had no effect on cell-surface hydrophobicity. Cells were treated with high concentrations of these compounds (relative to their MICs) for a short period of time to demonstrate their physical effect on the outer membrane and to minimize any physiological effect prolonged incubation with these antibacterial agents might have.

DISCUSSION

The possibility that quinolones may penetrate the outer membrane of gram-negative bacteria by additional, nonporin pathways was suggested by the reduction in MICs of quinolones for rough mutants of *Salmonella typhimurium* and the relatively small increase in MICs among porin-deficient mutants of *E. coli* (8). The outer membrane of enterobacteria is constructed in such a manner that lipopolysaccharide moieties prevent access to the phospholipid bilayer (4, 22). Magnesium ions further reduce access to the phospholipid bilayer by complexing with polyphosphate groups of adjacent lipopolysaccharide molecules (6).

Polycationic antibiotics such as aminoglycosides and polymyxin B expose the lipid bilayer by displacing magnesium or other divalent cations; hydrophobic compounds can then diffuse through the newly created hydrophobic patches. This is the "self-promoted" pathway of aminoglycoside uptake proposed by Hancock et al. (6). EDTA exposes patches of the lipid bilayer as a result of its strong ability to chelate divalent cations (13). Thus, both polycations and chelating agents remove magnesium from the outer membrane but by different mechanisms—displacement and complex formation. Both classes of compounds cause an increase in the permeability of the outer membrane (7, 13) and sensitivity to lysis by complement (21); in some cases large amounts of lipopolysaccharide are released from the outer membrane (12).

TABLE 5. Effects of different antibiotics on cell-surface hydrophobicity (P_s)

Compound added[a]	P_s	% Difference in P_s
None	0.477 + 0.054	0
Fleroxacin	0.643 + 0.059	+35
Fleroxacin + MgCl (10 mM)	0.421 + 0.076	−12
Gentamicin	0.661 + 0.103	+38
EDTA	0.620 + 0.01	+30
Polymyxin B nonapeptide	0.818 + 0.032	+71
Ampicillin	0.486 + 0.065	+2

[a] Fleroxacin, gentamicin, and ampicillin were used at 100 μg/ml; EDTA was used at 5.0 mM; and polymyxin B nonapeptide was used at 10 μg/ml.

We propose that quinolones interact with the outer membrane as chelating agents. The quinolone nucleus has adjacent carbonyls at C-2 and C-3 which form a potential divalent-cation-chelating site. Changes in the fluorescence of chelating compounds often indicate the binding of metals and have been used to measure levels of cations in biological systems (19). Accordingly, changes in the fluorescence of fleroxacin were seen with divalent, but not monovalent, cations (Fig. 2). Outer-membrane perturbations typical of those seen with gentamicin and EDTA were seen in quinolone-treated cells. These include an increase in the permeability of the outer membrane (Table 4), sensitization of treated cells to lysis by low concentrations of SDS (Fig. 3), an increase in the hydrophobicity of the cell surface (Table 5), and the release of large amounts of endotoxin (lipopolysaccharide) by treated cells (16). These quinolone-induced alterations, as well as the antibacterial action of the drug (Table 2), are prevented or ameliorated by magnesium. Magnesium has identical effects on the action of aminoglycosides and EDTA on the outer membrane (5, 7).

Excess magnesium prevents quinolones from chelating lipopolysaccharide-associated magnesium and creating hydrophobic patches on the cell surface and thus limits their diffusion across the outer membrane to the porin pathway. Diffusion through the water-filled porins is influenced by the hydrophobicity of the compound; the less hydrophobic a compound is, the better it diffuses (18). The inverse relationship would apply to diffusion through a hydrophobic lipid bilayer; i.e., hydrophobic compounds would diffuse better than hydrophilic ones. Since all of the fluoroquinolones appear to permeabilize the outer membrane equally well (J. S. Chapman, unpublished results), the extent to which magnesium antagonizes the inhibitory action of a given quinolone should reflect the degree to which that compound uses the nonporin pathway for entry, which in turn is dependent on the hydrophobicity of the quinolone. This can be most clearly demonstrated in strain CS1293, in which the competing porin pathways have been eliminated. There is a positive correlation between the increasing hydrophobicity

of a quinolone and the size of the MIC increase in the presence of magnesium (Table 3). Hirai et al. (8) noted a similar relationship in the decrease in MICs for rough mutants as compared with MICs for the wild-type strain; the more hydrophobic the compound, the larger the decrease in the MIC.

The complex kinetics of fleroxacin uptake shown in Fig. 1 can be interpreted in terms of the chelating ability of quinolones. The immediate and rapid rise in cell-associated fleroxacin could be the result of fleroxacin chelating outer-membrane-bound magnesium and is reduced by adding free magnesium. The decrease in cell-associated fleroxacin may be the result of the quinolone-magnesium complex dissociating from the outer membrane; the rate of dissociation is independent of the concentration of magnesium in the buffer. As the quinolone-magnesium complex dissociates from the outer membrane, the phospholipid bilayer becomes accessible to hydrophobic compounds. Cells become susceptible to SDS shortly after the decrease in cell-associated fleroxacin (compare Fig. 1 and 3). The third phase of uptake is the energy-independent, nonsaturable diffusion of fleroxacin through the outer membrane and the cytoplasmic membrane to the cell interior. The diffusion of fleroxacin through the outer membrane occurs through both porin and nonporin pathways; the relative contribution of each to total uptake is influenced by the hydrophobicity of the quinolone. The increase seen in the MIC in the presence of magnesium implies that the nonporin pathway is operational and significant at the low concentrations needed to inhibit growth.

The previously reported energy independence of quinolone uptake (Hooper et al., 26th ICAAC) (1) was confirmed in our experiments with dinitrophenol-poisoned cells and the active-transport-deficient strain MAL300. This is in contrast to aminoglycoside uptake, which is multiphasic, where entry through the cytoplasmic membrane is an active-transport process.

The concentrations of quinolones used to demonstrate the outer-membrane effects might be considered nonphysiological in light of the low MICs reported for quinolones in *E. coli* (Table 2). However, the fleroxacin-induced outer membrane effects are caused by concentrations of fleroxacin that are achievable in human sera (maximum concentration in serum, 6.1 μg/ml [24]) and can be considered physiologically relevant in terms of host-parasite relationships. The exposure of hydrophobic domains on the cell surface by polymyxin B results in lysis by complement of otherwise resistant gram-negative bacteria (21). It has recently been demonstrated that growth of *Klebsiella pneumoniae* in the presence of sub-MICs of ciprofloxacin results in increased binding of complement component C3 to the bacterial surface (23). Thus, quinolones may contribute to the elimination of pathogens by rendering them susceptible to components of the host immune system.

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