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In Vitro Antibacterial Activities of Tosufloxacin against and Uptake of Tosufloxacin by Outer Membrane Mutants of *Escherichia coli*, *Proteus mirabilis*, and *Salmonella typhimurium*

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The antibacterial activities of tosufloxacin and other quinolones against and apparent uptakes of tosufloxacin and other quinolones by outer membrane mutants of *Escherichia coli*, *Proteus mirabilis*, and *Salmonella typhimurium* were studied. The hydrophobicity of tosufloxacin was nearly equal to that of ofloxacin or lower than those of sparfloxacin and nalidixic acid. OmpF- and OmpC-deficient *E. coli* and 40-kDa porin-deficient *P. mirabilis* mutants were twofold more susceptible to tosufloxacin and sparfloxacin but two- to fourfold less susceptible to other quinolones than their parent strains. In *S. typhimurium* lipopolysaccharide-deficient (rough) mutants, the differences in susceptibility to tosufloxacin were similar to those to sparfloxacin and nalidixic acid. The apparent uptake of tosufloxacin by intact cells was increased in porin-deficient mutants compared with that by their parent strain. These results suggest that the permeation route of tosufloxacin across the outer membrane is different from that of other fluoroquinolones and that tosufloxacin may permeate mainly through the nonporin pathway, presumably phospholipid bilayers. However, this characteristic is independent of the hydrophobicity of the molecule.

The mechanisms of quinolone resistance in gram-negative bacteria, especially *Escherichia coli*, have been well studied. One of the major resistance mechanisms is alteration of the subunit structure of DNA gyrase (1, 5, 10, 12, 14, 16, 20, 22, 27, 28, 30, 31), and another is decreased permeability through the outer membrane from a reduced number of porins (3, 4, 6–8, 11, 13–15). In *E. coli*, fluoroquinolones are thought to cross the outer membrane primarily through the OmpF porin; this has been demonstrated previously with defined porin-deficient strains (4, 6, 13, 26). Moreover, the lipopolysaccharide (LPS)-deficient (rough) mutants were more susceptible to hydrophobic quinolones than was the wild-type strain, suggesting that the passage of quinolones through the outer membrane is not limited to porins (13).

Tosufloxacin is a fluoroquinolone that has potent activities against both gram-positive and gram-negative bacteria (9). We unexpectedly observed increased susceptibilities of *E. coli* and *Proteus mirabilis* porin-deficient mutants to tosufloxacin compared with those of their parent strains. In this study, we characterize tosufloxacin uptakes by wild-type strains and by porin-deficient mutants. Our results suggest that the mechanism of uptake for tosufloxacin is different from that for other quinolones and that tosufloxacin may permeate the outer membrane mainly through a nonporin pathway, presumably phospholipid bilayers.

MATERIALS AND METHODS

Materials. Tosufloxacin (Toyama Chemical Co., Ltd., Toyama, Japan), ofloxacin and nalidixic acid (Daiichi Seiyaku Co., Ltd., Tokyo, Japan), ciprofloxacin (Bayer Yakuhin Co., Ltd., Osaka, Japan), norfloxacin (Kyorin Seiyaku Co., Ltd., Tokyo, Japan), cefoxitin (Banyu Seiyaku Co., Ltd., Tokyo, Japan), erythromycin (Shionogi Seiyaku Co., Ltd., Osaka, Japan), and sparfloxacin (Dainihon Seiyaku Co., Ltd., Tokyo, Japan) were all commercially available. Other reagents were all analytical grade and commercially available.

Bacterial strains. The source and characteristic(s) of each strain used are described in Table 1. *E. coli* KY strains and *E. coli* KL-16 were derived from *E. coli* K-12, the parent strain. *E. coli* KY-2563 (wild type), KY-2562 ($OmpF^-$ $OmpC^-$), KY-2209 ($OmpF^+$ $OmpC^-$), and KY-2201 ($OmpF^-$ $OmpC^+$) were kindly provided by T. Yura (23), Kyoto University, Kyoto, Japan. *E. coli* KL-16 was used for the preparation of DNA gyrase. The series of LPS-deficient mutants of *Salmonella typhimurium* (Table 2) was kindly provided by Lindberg and Hellerqvist (17) and Roantree et al. (21). *P. mirabilis* T-116 was a clinical isolate which showed susceptibility to quinolones. A spontaneous porin-deficient mutant of *P. mirabilis* T-116 was isolated on the basis of its cefoxitin resistance (19, 24). The cefoxitin concentration used for the isolation of this porin-deficient mutant was 25 μg/ml for *P. mirabilis* T-116, and the mutant lacking porins was termed *P. mirabilis* T-116R1. The isolation frequency of this mutant was about 5×10^{-8} to 1×10^{-9} CFU per plate.

Determination of susceptibility. MICs were determined by a twofold serial agar dilution method. An overnight culture of the bacterial strain in heart infusion broth (Difco Laboratories) was diluted 100-fold with fresh medium, and 5 μl of the bacterial suspension (about 2×10^6 cells per ml) was inoculated onto Mueller-Hinton agar (Difco Laboratories) plates by using a Microplanter (Sakuma Factory, Tokyo, Japan). The plates were measured after incubation at 37°C for 18 h. The MIC was the lowest concentration of the drug that inhibited the development of visible growth on agar.

Inhibitory effect on DNA gyrase. DNA gyrase was pre-

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TABLE 1. Bacterial strains

Strain	Characteristic(s)	Source (reference)
KY-2563	*thi tsx malA*	T. Yura (23)
KY-2562	KY-2563 *ompB101* ($OmpC^-$ $OmpF^-$)	T. Yura
KY-2201	KY-2563 *ompB101 ompCp*$_1$ ($OmpF^-$)	T. Yura
KY-2209	KY-2563 *ompB101 ompFp*$_9$ ($OmpC^-$)	T. Yura
T-116	Clinical isolate	This laboratory
T-116R1	Mutant of T-116 selected for cefoxitin resistance	This laboratory
LT2	Autotroph (smooth)	Lindberg (17)
SL1034	*rfe465*(SR)	Roantree (21)
TV119	*rfb430*(Ra)	Roantree
SL733	*rfaK*(Rb_1)	Roantree
TV160	*metA22 hryB2 galT411 rfa418* (Rb_2)	Lindberg
TV148	*rfa433*(Rb_3)	Lindberg
LT2M1	*galE*(Rc)	Roantree
SL1004	*rfb430 rfaG571*(Rd_1)	Roantree
TA2168	*hisC3076 galE506 rfa1009*(Re)	Lindberg

pared from *E. coli* KL-16 and *P. mirabilis* T-116 by the method of Fujimaki et al. (9). Each enzyme fraction was stored in a refrigerator at −40°C. The inhibition of DNA gyrase supercoiling activity was assayed in a manner similar to that described previously (9). The standard reaction mixture containing subunit A and B proteins, a drug solution, and relaxed pBR322 DNA was incubated at 37°C for 2 h. The reaction was stopped by the addition of proteinase K (Sigma) to a final concentration of 1% and was subjected to agarose gel electrophoresis (0.8% agarose in 40 mM Tris-acetate–2 mM EDTA; 3 V/cm for 16 h). The gel was stained with ethidium bromide (0.5 μg/ml) for 1 h and photographed with a shortwave UV lamp (254 nm). The negatives were traced with a densitometer (GS300 Transmittance/Reflectance Scanning Densitometer; Hoefer Scientific Instruments, San Francisco, Calif.).

Measurement of quinolone uptake. The apparent uptakes of quinolones were measured by a modification of the methods of Chapman and Georgopapadakou (6) and Hirai et al. (13, 14). Bacterial cells were grown with Antibiotic Medium 3 (Difco Laboratories) to an A_{660} of 0.6, harvested by centrifugation, washed once with 50 mM sodium phosphate buffer–0.145 M NaCl (pH 7.0), and suspended at an A_{660} of 12 in the same buffer. The concentrations of cells at this stage were about 4×10^9 to 6×10^9 CFU/ml. Quinolones were added to a final concentration of 10 μg/ml, and the 0.5 ml of cell suspension was incubated for 10 min at 37°C. Then, the cell suspension was diluted immediately for 2.0 ml of buffer. The cells were centrifuged (10,000 × *g* for 1 min) and washed once with 2.0 ml of buffer. The cell pellets obtained were extracted four times with a 1-ml portion of 100% acetonitrile, and the extracts were evaporated to dryness under reduced pressure by using a VC-960 Centrifugal Concentrator (TAITEC Co., Ltd., Tokyo, Japan). The

TABLE 2. Chemical structure of the LPS of *S. typhimurium*

Strain	Chemical structure[a]
LT2(S)	[Abe / Man-Rha-Gal]$_n$-Man-Rha-Gal-Glu-Gal-Glu-Hep-Hep-KDO-KDO-lipid A
SL1034(SR)	Abe / Man-Rha-Gal-Glu-Gal-Glu-Hep-Hep-KDO-KDO-lipid A
TV119(Ra)	GNac / Glu-Gal-Glu-Hep-Hep-KDO-KDO-lipid A
SL733(Rb_1)	Gal / Glu-Gal-Glu-Hep-Hep-KDO-KDO-lipid A
TV160(Rb_2)	Gal / Gal-Glu-Hep-Hep-KDO-KDO-lipid A
TV148(Rb_3)	Gal / Glu-Hep-Hep-KDO-KDO-lipid A
LT2M1(Rc)	Hep / Glu-Hep-Hep-KDO-KDO-lipid A
SL1004(Rd_1)	Hep / Hep-Hep-KDO-KDO-lipid A
TA2168(Re)	KDO / KDO-KDO-lipid A

[a] Abe, abequose; Man, mannose; Rha, rhamnose; Gal, galactose; Glu, glucose; Hep, heptose; GNac, *N*-acetylglucosamine; KDO, 2-keto-3-deoxyoctonic acid.

evaporated extracts were dissolved with 0.2 ml of the solvent for high-pressure liquid chromatography (HPLC). The solvent was made up of acetonitrile–1 M citric acid disodium–10% methansulfonic acid–10% triethylamine–H_2O (18:3:2:2:75 for ofloxacin and norfloxacin, 20:3:2:2:73 for ciprofloxacin, 25:3:2:2:68 for tosufloxacin and sparfloxacin, and 40:3:2:2:53 for nalidixic acid). The amount of each quinolone in the resulting extract was defined as the uptake and expressed as nanograms of quinolone per milligram (dry weight) of cell. The concentration of each quinolone was determined by HPLC under the following conditions: an Inertsil ODS-2 column (Gasukuro Kogyo Co., Ltd., Tokyo, Japan) (precolumn, 4 by 50 mm; analytical column, 4 by 150 mm), a flow rate of 1.0 ml/min, and detection wavelengths of 286 nm (ofloxacin), 283 nm (norfloxacin), 271 nm (ciprofloxacin), 345 nm (tosufloxacin), 289 nm (sparfloxacin), and 320 nm (nalidixic acid). Twenty-five microliters of sample was injected into the column.

Measurement of amounts of outer membrane protein. The amounts of outer membrane protein in total protein of whole cells were measured and expressed as micrograms of outer membrane protein per milligram of total protein of whole cells. Outer membrane proteins were prepared as 2% *N*-lauroylsarcosine-insoluble fractions by the method of Sawai et al. (24). Protein amounts were determined according to the method of Lowry et al. (18).

The porin contents of total outer membrane protein were measured by densitometric tracing of sodium dodecyl sulfate (SDS)–polyacrylamide gels. A 10-μg portion of outer membrane protein was analyzed by SDS–10% polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue R250 (Sigma). The gels were traced with a densitometer (GS-300 Transmittance/Reflectance Scanning Densitometer; Hoefer), and porin contents were expressed as the percent of porin in total outer membrane protein.

Cell surface hydrophobicity. Cell surface hydrophobicity was determined by the method of Hirai et al. (14) with slight modifications. Bacterial cells were grown in Antibiotic Medium 3 (Difco) to an A_{660} of 0.6, harvested by centrifugation, washed once with 50 mM phosphate buffer (pH 7.0), and suspended at an A_{530} of 1.0. A 5-ml sample of bacterial cells was mixed with 10 ml of *p*-xylene by vortexing for 2 min and then was allowed to separate at room temperature for 20 min. The cell surface hydrophobicity was calculated as (A_{530} of untreated control − A_{530} of treated sample)/A_{530} of untreated control.

Quinolone hydrophobicities. The hydrophobicities of the quinolones were determined by partitioning with 0.1 M phosphate buffer (pH 7.2) and *n*-octanol by the methods of Hirai et al. (13) and Chapman and Georgopapadakou (6). The buffer-octanol mixture was incubated for 24 h to equilibration, and then each quinolone was added to a final concentration of 10 μg/ml in the aqueous phase. The partition coefficient is expressed as the ratio of the quinolone concentration in the organic phase to that in the aqueous phase. Quinolone concentrations were determined by measuring UV A_{286} (ofloxacin), A_{283} (norfloxacin), A_{271} (ciprofloxacin), A_{345} (tosufloxacin), A_{289} (sparfloxacin), and A_{320} (nalidixic acid).

SDS-PAGE. Outer membrane proteins were prepared by the method of Sawai et al. (24). The OmpF and OmpC proteins in *E. coli* were analyzed with an 8 M urea–SDS–10% polyacrylamide gel as described by Uemura and Mizushima (25). The outer membrane proteins of *P. mirabilis* were analyzed with an SDS-10% polyacrylamide gel. (24).

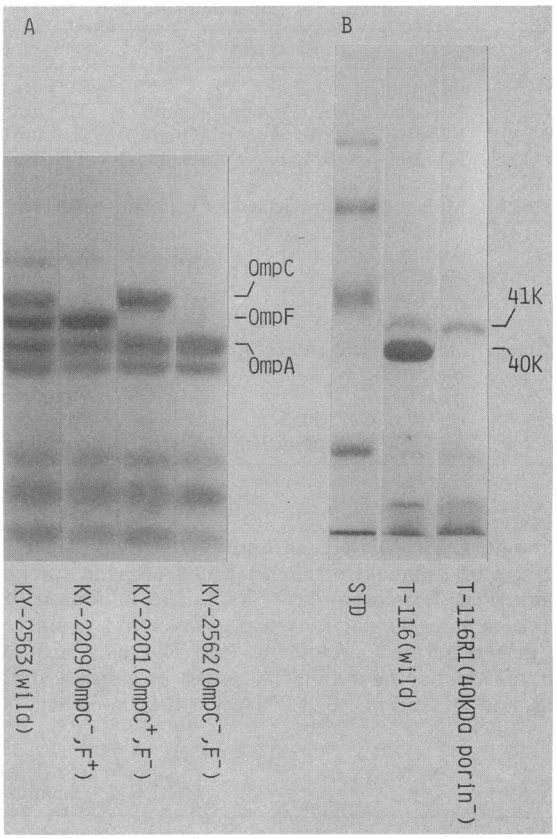

FIG. 1. Outer membrane proteins from *E. coli* and *P. mirabilis* separated by SDS-PAGE. (A) 8 M urea-SDS-10% polyacrylamide gel; (B) SDS-10% polyacrylamide gel. The strains are listed underneath the lanes. Lane STD contained molecular mass standards that correspond to, from top to bottom, myosin (200 kDa), phospholipase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and α-chymotrypsinogen (25.7 kDa).

RESULTS

Strain characterization. The outer membrane profiles of *E. coli* KY series strains by SDS-PAGE are shown in Fig. 1A. The outer membrane profiles of porin-deficient mutants were unaltered compared with those of the wild-type strain except for reduced numbers of the OmpF or OmpC porin (data not shown).

The porin-deficient mutant *P. mirabilis* T-116R1 was isolated by measuring its cefoxitin resistance. We have already reported that *P. mirabilis* had only one kind of porin protein, a protein with a molecular mass of 40 kDa (19). The outer membrane profiles of *P. mirabilis* T-116R1 were unaltered except for the lack of the 40-kDa protein (Fig. 1B).

The 50% inhibitory concentrations needed for inhibition of the supercoiling activities of *E. coli* KL-16 and *P. mirabilis* T-116 DNA gyrase are listed in Table 3. The DNA gyrase obtained from *P. mirabilis* T-116 was about two- to threefold less susceptible to quinolones than that from *E. coli* KL-16. Tosufloxacin was a potent inhibitor compared with other quinolones.

Quinolone susceptibilities of outer membrane mutants. The bacterial susceptibilities of outer membrane mutants of *E. coli*, *P. mirabilis*, and *S. typhimurium* to quinolones and other antimicrobial agents are shown in Table 4. Decreases

TABLE 3. Inhibitory effects of quinolones against DNA gyrase from *E. coli* KL-16 and *P. mirabilis* T-116

Compound[a]	MIC (μg/ml)		ID_{50} (μg/ml)[b]	
	E. coli KL-16	*P. mirabilis* T-116	*E. coli* KL-16	*P. mirabilis* T-116
NFLX	0.05	0.2	0.80	1.85
CPFX	0.0125	0.025	0.35	0.97
TFLX	0.025	0.1	0.28	0.75
OFLX	0.05	0.1	0.70	1.78
SPFX	0.025	0.2	0.80	1.00
NA	3.13	6.25	50	NT[c]

[a] NFLX, norfloxacin; CPFX, ciprofloxacin; TFLX, tosufloxacin; OFLX, ofloxacin; SPFX, sparfloxacin; NA, nalidixic acid.
[b] 50% inhibitory dose of DNA gyrase.
[c] NT, not tested.

in bacterial susceptibilities to cefoxitin were observed with *E. coli* and *P. mirabilis* porin-deficient mutants but not with *S. typhimurium* LPS stepwise-deficient mutants. Cefoxitin was believed to mainly pass through porins.

Increases in bacterial susceptibilities to erythromycin, novobiocin, and crystal violet were observed with *S. typhimurium* LPS stepwise-deficient mutants but not with *E. coli* and *P. mirabilis* porin-deficient mutants. These compounds were more hydrophobic than quinolones and susceptible to the effect of an LPS barrier. These results suggest that the composition of porins in *S. typhimurium* LPS-deficient mutants and the LPS structure of porin-deficient mutants were unaltered between the wild-type strains and their mutants.

The susceptibilities of the OmpF-deficient mutant KY-2201 and the OmpC-deficient mutant KY-2209 to tosufloxacin and sparfloxacin were equal to those of wild-type KY-2563, but the susceptibility of strain KY-2562, which lacks both OmpC and OmpF, was twofold higher than that of KY-2563. The susceptibilities of strains KY-2201 and KY-2562 to norfloxacin, ciprofloxacin, and ofloxacin were twofold lower than those of KY-2563, and the susceptibilities of strain KY-2209 to these quinolones were equal to those of KY-2563. The susceptibilities of all porin-deficient mutants to nalidixic acid were equal to that of KY-2563. As with *E. coli* porin-deficient mutants, the susceptibilities to tosufloxacin and sparfloxacin of *P. mirabilis* T-116R1, which lacks the 40-kDa porin, were twofold higher than those of its wild type, T-116, but the susceptibilities of T-116R1 to norfloxacin, ciprofloxacin, and ofloxacin were two- to fourfold lower than those of T-116. The differences in the susceptibilities of porin-deficient mutants and those of their wild-type strains appeared stronger for *P. mirabilis* than for *E. coli*.

The susceptibilities of LPS stepwise-deficient mutants of *S. typhimurium* to norfloxacin and ciprofloxacin were not affected by alteration of the LPS structure. These mutants showed a slight increase in susceptibility to ofloxacin compared with that of the parent strain. In contrast, the susceptibilities of LPS-deficient mutants (Rd_1 and Re) to tosufloxacin were fourfold lower than that of wild-type LT2, and the relative activities of tosufloxacin against LPS-deficient mutants were comparable to those of sparfloxacin and nalidixic acid. Nevertheless, the hydrophobicity of tosufloxacin was similar or slightly lower than that of ofloxacin, and the increase in its antibacterial activity against LPS-deficient mutants was larger than that of ofloxacin.

Uptake of tosufloxacin. Increased susceptibilities of porin-deficient mutants to tosufloxacin might result from increased drug uptake. We examined the accumulation of tosufloxacin and other quinolones by wild-type *E. coli* KY-2563 and *P. mirabilis* T-116 and their derivatives KY-2562 and T-116R1. Under our assay conditions, the accumulations of drugs plateaued at 10 min after the addition of drugs (data not shown). The accumulation of drugs is shown as the apparent uptake when the uptake by the wild type is expressed as 100%.

TABLE 4. Antibacterial activities of antimicrobial agents[a] against outer membrane mutants of *E. coli*, *P. mirabilis*, and *S. typhimurium*

Strain	MIC (μg/ml)									
	NFLX (0.021)[b]	CPFX (0.027)	TFLX (0.29)	OFLX (0.31)	SPFX (0.89)	NA (3.38)	CFX	EM	NOVO	CV
E. coli										
KY-2563 (wild type)	0.05	0.0125	0.025	0.05	0.025	3.13	3.13	25	>200	25
KY-2209 ($OmpC^-$ $OmpF^+$)	0.05	0.0125	0.025	0.05	0.025	3.13	3.13	25	>200	25
KY-2201 ($OmpC^+$ $OmpF^-$)	0.1	0.025	0.025	0.1	0.025	3.13	25	25	>200	25
KY-2562 ($OmpC^-$ $OmpF^-$)	0.1	0.025	0.0125	0.1	0.0125	3.13	50	25	>200	25
P. mirabilis										
T-116 (wild type)	0.2	0.025	0.1	0.1	0.2	6.25	6.25	200	50	3.13
T-116R1 (Por^-)[c]	0.78	0.1	0.05	0.39	0.1	12.5	50	200	50	3.13
S. typhimurium										
LT2(S)	0.1	0.0125	0.025	0.1	0.025	6.25	3.13	50	>200	100
SL1034(SR)	0.1	0.0125	0.025	0.1	0.025	6.25	3.13	50	>200	25
TV119(Ra)	0.1	0.0125	0.0125	0.1	0.0125	6.25	3.13	50	200	12.5
SL733(Rb_1)	0.1	0.0125	0.025	0.1	0.025	6.25	3.13	50	>200	12.5
TV160(Rb_2)	0.1	0.0125	0.025	0.1	0.025	6.25	3.13	50	>200	12.5
TV148(Rb_3)	0.1	0.0125	0.0125	0.05	0.00625	3.13	3.13	50	>200	12.5
LT2M1(Rc)	0.1	0.0125	0.0125	0.05	0.00625	3.13	3.13	50	200	6.25
SL1004(Rd_1)	0.1	0.0125	0.00625	0.05	0.00313	1.56	3.13	6.25	50	0.78
TA2168(Re)	0.1	0.0125	0.00625	0.05	0.00313	1.56	3.13	1.56	25	0.39

[a] NFLX, norfloxacin; CPFX, ciprofloxacin; TFLX, tosufloxacin; OFLX, ofloxacin; SPFX, sparfloxacin; NA, nalidixic acid; CFX, cefoxitin; EM, erythromycin; NOVO, novobiocin; CV, crystal violet.
[b] Numbers in parentheses are the partition coefficients obtained with *n*-octanol–50 mM phosphate buffer (pH 7.2). For details, see Materials and Methods.
[c] Porin-deficient mutant.

TABLE 5. Apparent uptakes of quinolones by outer membrane mutants of *E. coli* and *P. mirabilis*

Strain	Apparent uptake (ng/mg [dry cell])[a]					
	NFLX	CPFX	OFLX	TFLX	SPFX	NA
E. coli						
KY-2563 (wild type)	60.30 ± 10.98 (100)[b]	37.39 ± 4.85 (100)	11.37 ± 1.07 (100)	9.45 ± 0.64 (100)	4.10 ± 1.16 (100)	18.63 ± 2.46 (100)
KY-2562 (OmpC$^-$ OmpF$^-$)	28.36 ± 7.62 (47)	22.22 ± 6.89 (59)	4.96 ± 0.24 (44)	11.92 ± 0.48 (126)	4.88 ± 0.56 (119)	13.04 ± 0.49 (70)
P. mirabilis						
T-116 (wild type)	27.78 ± 7.78 (100)	20.78 ± 1.47 (100)	12.28 ± 1.37 (100)	4.80 ± 0.68 (100)	2.53 ± 0.67 (100)	26.71 ± 4.76 (100)
T-116R1 (Por$^-$)[c]	7.99 ± 2.06 (29)	6.78 ± 3.05 (33)	5.38 ± 0.17 (44)	7.53 ± 1.16 (157)	3.61 ± 0.54 (143)	7.48 ± 2.10 (28)

[a] The values are means ± standard deviations of five separate experiments. For abbreviations, see Table 3, footnote *a*.
[b] Values in parentheses are relative to the uptake of the wild-type strain, expressed as 100%.
[c] Porin-deficient mutant.

Tosufloxacin was more efficiently accumulated by the OmpC- and OmpF-deficient mutant KY-2562, with the uptake being approximately 130% compared with that by the wild-type KY-2563, whereas the uptakes of ofloxacin, norfloxacin, and ciprofloxacin by KY-2562 were approximately 50% compared with those by the wild type (Table 5). Similarly, the results for uptakes of tosufloxacin and sparfloxacin were approximately 120% compared with those by the wild type. However, for the most hydrophobic quinolone, nalidixic acid, the uptake was decreased to approximately 70% compared with that by the wild-type strain.

For *P. mirabilis*, the difference in uptake by the porin-deficient mutant and that by the wild-type strain was much larger than that for *E. coli*. The 40-kDa porin-deficient mutant T-116R1 also accumulated approximately 60% more tosufloxacin than did wild-type T-116, but the uptakes of norfloxacin, ciprofloxacin, ofloxacin, and nalidixic acid were decreased to 28 to 44% of those by the wild-type strain. As with *E. coli* and its uptake of sparfloxacin, T-116R1 accumulated approximately 40% more sparfloxacin than did the wild type.

Cell surface hydrophobicities of outer membrane mutants. It is still unknown why porin deficiency enhanced the intracellular accumulation of tosufloxacin and sparfloxacin. The direct exposure of the phospholipid bilayers in the outer membrane to an outside environment, when porins are lacking, has been suggested by Yamaguchi et al. (29). Furthermore, the pH-dependent binding of ciprofloxacin and enoxacin to liposomes predominantly containing phospholipids, which could be the first step in the diffusion of these compounds across the lipid bilayers, has been reported by Bedard and Bryan (2). Presumably, tosufloxacin may also associate with and then permeate the phospholipid bilayers exposed to the outside environment in the outer membrane. To confirm our conclusion, we examined the effect of porin deficiency on the outer membrane by measuring cell surface hydrophobicity in intact cells (Table 6). In the control experiment, the cell surface hydrophobicities of *S. typhimurium* LPS-deficient mutants were increased stepwise by the lack of LPS. However, contrary to our expectations, cell surface hydrophobicity was increased in the *P. mirabilis* porin-deficient mutant (about 158%) and unaltered in the *E. coli* porin-deficient mutant.

Determination of amounts of outer membrane protein in whole cells. The lack of differences in the hydrophobicity of the porin-deficient mutant and that of the wild-type strain in *E. coli* suggests that the amount of outer membrane protein in the total protein of whole cells might be smaller than that in *P. mirabilis*. We determined the amounts of outer membrane protein in the total protein in whole cells of *E. coli* KY-2563 and KY-2562 and *P. mirabilis* T-116 and T-116R1 (Table 7). For the wild-type strains, the amount of outer membrane protein in *P. mirabilis* T-116 was about twofold larger than that in *E. coli* KY-2563. However, we observed a significant decrease in the amount of outer membrane protein in *P. mirabilis* T-116R1 but not in *E. coli* KY-2562. Decreased amounts of outer membrane protein might result from a deficiency in porins. We examined the porin content of total outer membrane proteins. In *E. coli*, the porin content (OmpF and OmpC) was about 38% of the total outer membrane, whereas the 40-kDa porin of *P. mirabilis* occupied about 78% of the total outer membrane proteins. These values are consistent with the reduced amounts of outer membrane protein in porin-deficient mutants.

DISCUSSION

In this study, we determined the outer membrane permeation of tosufloxacin by using an isogenic set of mutants with altered outer membrane structures.

Inhibitory doses of quinolones against DNA gyrase from *P. mirabilis* were about two- to threefold less than those of quinolones against DNA gyrase from *E. coli*. This difference

TABLE 6. Cell surface hydrophobicities of outer membrane mutants

Strain	Hydrophobicity[a]
E. coli	
KY-2563 (wild type)	0.84 (100)[b]
KY-2201 (OmpC$^-$)	0.81 (96)
KY-2209 (OmpF$^-$)	0.87 (104)
KY-2562 (OmpC$^-$ OmpF$^-$)	0.85 (101)
P. mirabilis	
T-116 (wild type)	0.34 (100)
T-116R1 (40-kDa Por$^-$)[c]	0.57 (168)
S. typhimurium	
LT2(S)	0.26 (100)
TV119(Ra)	0.32 (123)
TV160(Rb_2)	0.50 (192)
LT2M1(Rc)	0.57 (219)
TA2168(Re)	0.82 (315)

[a] Values are partition coefficients with *p*-xylene–cell suspension. See Materials and Methods for details.
[b] Values in parentheses are relative to that for the wild type (100%) and are expressed as percents.
[c] Porin-deficient mutant.

TABLE 7. Amounts of outer membrane proteins in total proteins of whole cells and porin contents of total outer membrane proteins

Strain	Amt of outer membrane proteins[a]	Porin content (%)[b]
E. coli		
KY-2563 (wild type)	17.32 (100)[c]	38 (OmpC OmpF)
KY-2562 (OmpC$^-$ OmpF$^-$)	11.74 (68)	
P. mirabilis		
T-116 (wild type)	31.75 (100)	78 (40-kDa porin)
T-116R1 (Por$^-$)[d]	5.74 (18)	

[a] The values are the means of three separate experiments and are expressed as micrograms per milligram of total protein.
[b] Values are expressed as percents of total outer membrane proteins.
[c] Numbers in parentheses are values relative to that for the wild-type strain (100%).
[d] Porin-deficient mutant.

is compatible with the difference in susceptibilities of *E. coli* and *P. mirabilis*. The susceptibility of *P. mirabilis*, which is less than that of *E. coli*, might be caused by the lack of susceptibility of DNA gyrase against quinolones.

Hirai et al. (13) reported that alteration of the LPS structure significantly affected outer membrane permeability to hydrophobic quinolones that had partition coefficients higher than 2.0 but not that to hydrophilic quinolones with partition coefficients lower than 1.0.

However, despite the fact that the hydrophobicity of tosufloxacin (0.29) is nearly equal to that of ofloxacin (0.30) and lower than those of sparfloxacin (0.89) and nalidixic acid (3.38), the susceptibility of *S. typhimurium* TA2168(Re) to tosufloxacin was fourfold higher than that of the parent strain, LT2(S). Similar findings have been noted for sparfloxacin and nalidixic acid. The outer membrane permeation of tosufloxacin was more affected by the barrier of LPS than we predicted from its hydrophobicity. Similarly, it has been previously noted that the activity of amifloxacin, which is more hydrophobic than nalidixic acid, was affected little by alterations in the LPS structure (26). These findings together with our results suggest that the permeation of quinolones through a barrier of LPS, at least in part, is not limited simply by the apparent hydrophobicity of the molecules but also by the differences in basal structure (quinoline, naphthyridine, or benzoxazine nucleus) or in each specific moiety.

Our result showed that a deficiency of the OmpF porin in an isogenic set of mutants caused reduced susceptibilities to norfloxacin, ciprofloxacin, and ofloxacin. OmpC porin deficiency had no apparent effect on susceptibility. These findings are in agreement with some results previously reported for norfloxacin (13, 14, 16) and enoxacin (4) and indicate that these hydrophilic quinolones pass through the outer membrane via the OmpF porin. We observed a similar relationship with the *P. mirabilis* 40-kDa porin-deficient mutant. However, despite the fact that the antibacterial activities of hydrophilic fluoroquinolones against porin-deficient mutants were decreased, the antibacterial activities of tosufloxacin and sparfloxacin were enhanced in porin-deficient mutants compared with those in the parent strains. These results suggest that tosufloxacin and sparfloxacin may interact with the outer membrane mainly by the nonporin pathways and that the lack of porins enhances the interaction or permeation of these drugs. Our conclusion is supported by the results obtained by measuring the uptakes of tosufloxacin and sparfloxacin by porin-deficient mutants. Consequently, these drugs were more efficiently accumulated by *E. coli* and *P. mirabilis* porin-deficient mutants than by the parent strains. These results suggest that the uptakes of tosufloxacin and sparfloxacin are different from those of other hydrophilic fluoroquinolones and that these drugs may permeate the outer membrane mainly through the phospholipid bilayers.

The susceptibility to nalidixic acid, which has more hydrophobicity than tosufloxacin or sparfloxacin, of *S. typhimurium* TA2168(Re) was increased compared with that of the parent strain, LT2(S). Moreover, in the uptake study, the accumulation of nalidixic acid in porin-deficient mutants was decreased compared with that in wild-type strains, suggesting that nalidixic acid can pass through the outer membrane via porin channels. This result suggests that nalidixic acid uses the porin channels to permeate the outer membrane, though the permeation was subject to the effect of the LPS barrier because of the high hydrophobicity of the molecule.

Under our assay conditions, an accumulation of drugs seems to be the total amount of drug incorporated into the cytoplasm and bound to the extracytoplasmic compartment, possibly the periplasm or the outer membrane. This conclusion is supported by previously measured uptakes of enoxacin, ciprofloxacin, and norfloxacin (3, 4, 15). Even the results of apparent uptake under these conditions reflect the susceptibilities of outer membrane mutants to quinolones, suggesting that the cell-associated drug plays an important role at least in part in determining susceptibility to quinolones. We could not find differences in the cell surface hydrophobicity of the *E. coli* porin-deficient mutant and that of the wild-type strain. Presumably, it is difficult to measure the precise and delicate cell surface hydrophobicity of porin-deficient mutant cells by using the *p*-xylene–aqueous phase partition coefficient system, because the exposure of phospholipid bilayers, by a lack of porins, to the outside environment might only limit local changes in the outer membrane.

The increase in the cell surface hydrophobicity of the *P. mirabilis* porin-deficient mutant, larger than that of *E. coli*, suggests that the effect of porin deficiency on outer membrane structure, probably exposure of the phospholipid bilayers, was strong in *P. mirabilis*. This conclusion was supported by our result that the amount of outer membrane protein in *P. mirabilis* was twofold larger that that in *E. coli* and that the porin content of the total outer membrane protein is much higher in *P. mirabilis* (78% of total outer membrane proteins) than in *E. coli* (38%).

At present, it is still not known in detail why or how tosufloxacin permeates the outer membrane of porin-deficient mutants. We speculate that the divalent metal ion chelation properties of the molecules are important, leading to outer membrane destabilization and allowing the so-called self-promoted uptake of these particular quinolones (6).

The characteristic structural difference of tosufloxacin compared with other fluoroquinolones is a 2,4-difluorophenyl moiety of the 1.4-dihydro-4-oxo-1.8-naphthyridine nucleus. We now expect that this moiety may play an important and unique role in outer membrane permeation.

The fact that a loss of porin proteins results in increased susceptibility to tosufloxacin suggests that tosufloxacin could be an effective antimicrobial agent when altered outer membrane permeations have rendered other antibiotics ineffective. Further studies will be required in order to determine the precise mechanisms of penetration of tosufloxacin

through the outer membranes of porin-deficient mutants of gram-negative bacteria.

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