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Mechanisms of Clinical Resistance to Fluoroquinolones in *Enterococcus faecalis*

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About 10% of 100 clinical isolates of *Enterococcus faecalis* were resistant to ≥25 μg of norfloxacin, ofloxacin, ciprofloxacin, and temafloxacin per ml. In this study, the DNA gyrase of *E. faecalis* was purified from a fluoroquinolone-susceptible strain (ATCC 19433) and two resistant isolates, MS16968 and MS16996. Strains MS16968 and MS16996 were 64- to 128-fold and 16- to 32-fold less susceptible, respectively, to fluoroquinolones than was ATCC 19433; MICs of nonquinolone antibacterial agents for these strains were almost equal. The DNA gyrase from ATCC 19433 had two subunits, designated A and B, with properties similar to those of DNA gyrases from other gram-positive bacteria such as *Bacillus subtilis* and *Micrococcus luteus*. Inhibition of the supercoiling activity of the enzyme from ATCC 19433 by the fluoroquinolones correlated with their antibacterial activities. In contrast, preparations of DNA gyrase from MS16968 and MS16996 were at least 30-fold less sensitive to inhibition of supercoiling by the fluoroquinolones than the gyrase from ATCC 19433 was. Experiments that combined heterologous gyrase subunits showed that the A subunit from either of the resistant isolates conferred resistance to fluoroquinolones. These findings indicate that an alteration in the gyrase A subunit is the major contributor to fluoroquinolone resistance in *E. faecalis* clinical isolates. A difference in drug uptake may also contribute to the level of fluoroquinolone resistance in these isolates.

The fluoroquinolones, such as norfloxacin, ofloxacin, ciprofloxacin, and others, have increasingly been used clinically because of their potent and broad antibacterial activities (18, 43). Studies with *Escherichia coli* and other gram-negative bacteria have shown that fluoroquinolones exert their antibacterial activities by inhibiting DNA gyrase (43, 44), an essential bacterial topoisomerase which converts relaxed DNA to the supercoiled form (8, 40). Generally, the in vitro inhibitory effects of fluoroquinolones on the supercoiling activity of *E. coli* DNA gyrase closely correlate with their antibacterial activities (19, 24, 37), although the mechanisms which lead to fluoroquinolone-mediated bacterial killing are not fully understood (5, 19).

Even though the frequency of resistance to fluoroquinolones in vitro is much lower than those of nalidixic acid and oxolinic acid (22, 32, 43), the number of fluoroquinolone-resistant strains has increased clinically, especially with bacterial species such as *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Staphylococcus aureus* (32). The resistance to fluoroquinolones in gram-negative bacteria such as *E. coli* and *P. aeruginosa* has been shown to be associated with modification of DNA gyrase or alteration of outer membrane permeability (3, 17, 19, 22, 28, 32, 35, 43). For gram-positive bacteria, the role of DNA gyrase in the antibacterial action of fluoroquinolones has been unclear, although DNA gyrase has been partially purified from *Bacillus subtilis* (33, 39), *Micrococcus luteus* (27), and *S. aureus* (41). For example, Fu et al. (10) reported that fluoroquinolones are poor inhibitors of the supercoiling activity of *M. luteus* gyrase at or near the MICs. Both novobiocin and coumermycin A_1, which are known to be inhibitors of the B subunit of DNA gyrase (20), fully inhibited the gyrase. Georgopapadakou and Dix (14) reported similar observations on the relation between the inhibitory actions of fluoroquinolones against *S. aureus* gyrase and their MICs for *S. aureus*.

We have been surveying the susceptibilities of gram-positive bacteria to fluoroquinolones and have found that the MICs for *Enterococcus faecalis* isolates have gradually increased for clinical isolates. Although *E. faecalis* is resistant to the old quinolones (15), fluoroquinolones such as ciprofloxacin (4, 25) and temafloxacin (31) have promising activity for the treatment of urinary tract infections. However, the MICs of these fluoroquinolones for *E. faecalis* are too close to the concentrations achievable in plasma (30, 34), presumably contributing to the emergence of fluoroquinolone resistance in *E. faecalis*. *E. faecalis* DNA gyrase has not previously been characterized, and the mechanisms of resistance to fluoroquinolones have not been studied.

We report the purification and some properties of DNA gyrase from *E. faecalis* and the inhibitory effects of fluoroquinolones on the supercoiling activity of the gyrase from a quinolone-susceptible strain and two quinolone-resistant isolates. We also examined quinolone uptake by these strains.

MATERIALS AND METHODS

Bacterial strains. *E. faecalis* ATCC 19433 from the Episome Institute was used as a reference strain. Fluoroquinolone-resistant isolates, MS16968 and MS16996, were clinically isolated from urine in 1989 and identified to the species level by the method described in the *Manual of Clinical Microbiology* (9) and with the API 20 Strep system (API System S.A.). The strains were maintained at −70°C in 50% glycerol.

Antimicrobial susceptibility. MICs were determined by a twofold agar dilution method. One loopful (ca. 10^4 CFU) of a diluted culture broth was inoculated onto a modified

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Mueller-Hinton agar (Nissui Seiyaku Co., Ltd.) plate. The MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth after 18 h at 37°C.

Chemical agents. Novobiocin, dithiothreitol (DTT), tRNA of *E. coli* W, bovine serum albumin, spermidine, phenylmethylsulfonyl fluoride, mutanolysin, lysozyme, proteinase K, Brij 58, and ATP were purchased from Sigma Chemical Co. Topoisomerase I was from Bethesda Research Laboratories. Subunits A and B of *B. subtilis* DNA gyrase were purified from strain ATCC 6633 by the procedure of Orr and Staudenbauer (33). Subunits A and B of *E. coli* KL-16 DNA gyrase were purified as described previously (1, 38). Epoxy-activated Sepharose 6B and DEAE-Sepharose CL-6B were purchased from Pharmacia Fine Chemicals Co., Ltd. Plasmid pBR322 was purchased from Takara Shuzo Co., Ltd. Norfloxacin was obtained from Kyorin Pharmaceutical Co., Ltd.; ofloxacin was from Daiichi Pharmaceutical Co., Ltd.; ciprofloxacin was from Bayer Yakuhin Co., Ltd.; and temafloxacin was from Tanabe Seiyaku Co., Ltd. All other agents were obtained from their respective manufacturers.

Enzyme purification. *E. faecalis* cells were grown to the late log phase in 10 liters of tryptic soy broth (Difco Laboratories) supplemented with 3% glycine and harvested by centrifugation at 7,000 × *g* at 4°C. Cells were washed once with TGED buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA, 1 mM DTT, 10% [vol/vol] glycerol) and were stored at −70°C until use. The cell lysis and purification steps were carried out at 0 to 4°C unless indicated otherwise. The frozen cells (usually 40 g) were thawed with 150 ml of TGED buffer, 5 mM DTT–10 mM $MgCl_2$–0.4 M KCl–1 mM PMSF, 10 μg of mutanolysin per ml, and 1 mg of lysozyme per ml were successively added; and the cell suspension was incubated at 37°C for 1 h. Brij 58 (0.25%) was added, and after 15 min, the lysate was chilled to 0°C. After the lysate was sonicated with 30% (wt/vol) sea sand (E. Merck AG) for 10 min, the extract was centrifuged at 135,000 × *g* for 1.5 h in a Beckman type 42.1 rotor. Solid ammonium sulfate was added to the supernatant to give 70% saturation (0.47 g/ml); and after stirring for 1 h, the precipitate was collected by centrifugation at 100,000 × *g* for 20 min, suspended in 50 ml of TGED buffer (pH 8.0), and dialyzed against 3 liters of the same buffer for 12 h.

The dialysate was applied to a column of novobiocin-Sepharose (bed volume, 20 ml) previously equilibrated with TGED buffer. Novobiocin-Sepharose was prepared by coupling novobiocin to epoxy-activated Sepharose 6B as described by Staudenbauer and Orr (38). The column was washed with 120 ml of TGED buffer and eluted successively with 0.2 M KCl, 2 M KCl, 5 M urea, 2 M KCl–5 M urea, and 2 M KCl–5 M urea (pH 4.0). The A subunit eluted mainly with 2 M KCl in TGED buffer, and the B subunit eluted with 2 M KCl–5 M urea in TGED buffer. In accordance with the nomenclature of the subunits of *E. coli* gyrase (38), active fractions at 2 M KCl and 2 M KCl–5 M urea were designated subunits A and B of *E. faecalis* gyrase, respectively.

Active fractions of subunits A and B were pooled and immediately dialyzed against 3 liters of TGED buffer for 12 h. The dialysate of subunit A was applied to a column of DEAE-Sepharose CL-6B (bed volume, 10 ml) previously equilibrated with TGED buffer (0.025 M KCl), and the activity was eluted with 200 ml of a 0.025 to 0.5 M KCl linear gradient containing TGED buffer. The active fractions of subunit A eluted at around 0.2 M KCl. The dialysate of subunit B fractions was further purified by a second chromatography on novobiocin-Sepharose. The sample was applied to the column (bed volume, 5 ml) equilibrated with TGED buffer and eluted in a stepwise manner with 2 M KCl, 2.5 M urea, 5 M urea, 2 M KCl–5 M urea, and 2 M KCl–5 M urea (pH 4.0). The active fractions of subunit B eluted with 2 M KCl–5 M urea. The active fractions of subunit A and B proteins were dialyzed against TGED buffer, concentrated by dialysis against TGED buffer containing 50% (vol/vol) glycerol, and stored at −20°C. These preparations were stable during storage at −20°C for at least several months. The protein assay was performed by the procedure of Bradford (2).

DNA supercoiling assay and IC_{50} determinations. The assay of DNA gyrase subunits was modified from those described previously (13, 29). One unit of gyrase was defined as the amount of enzyme that catalyzed the conversion of one-half of 0.1 μg of relaxed closed circular pBR322 DNA to the supercoiled form in 1 h at 37°C, as discerned by agarose gel electrophoresis (13). Plasmid pBR322 was relaxed by topoisomerase I by the recommended protocol of the manufacturer. DNA gyrase supercoiling activity was assayed as described previously (11, 12, 37). Briefly, the standard reaction mixture (20 μl) contained 20 mM Tris hydrochloride (pH 7.5), 20 mM KCl, 2 mM $MgCl_2$, 2 mM spermidine, 2 mM ATP, 1 mM DTT, 10 μg of bovine serum albumin per ml, 30 μg of tRNA per ml, 0.1 μg of relaxed pBR322, drug solution, and DNA gyrase subunits A and B (1 U each). A control reaction without drug was included. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 3 μl of proteinase K (1 mg/ml), and then the reaction mixture was subjected to agarose gel electrophoresis (0.8% agarose in 40 mM Tris acetate–2 mM EDTA). The gel was stained in 0.5 μg of ethidium bromide per ml and photographed upon exposure to UV light. The negative was traced with a densitometer (CS-9000; Shimadzu Seisakusho Co., Ltd.). Densitometer tracings of negatives were used to determine the IC_{50}s. The IC_{50} was defined as the concentration of drug that inhibited 50% of the supercoiling activity of DNA gyrase in a standard reaction mixture.

Norfloxacin uptake by *E. faecalis*. The uptake of norfloxacin by intact cells was measured essentially as described by Hirai et al. (17). *E. faecalis* cells were grown to the late log phase in tryptic soy broth, and norfloxacin was added to the culture at a final concentration of 10 μg/ml. At various time intervals, 10 ml of the culture was rapidly chilled and washed once with 2 ml of ice-cold saline; the final pellet was suspended in 1 ml of 5% acetic acid solution. Cell-associated norfloxacin was extracted by heating the cells in a boiling-water bath for 7 min. The concentration of norfloxacin in the supernatant was quantitated at 270 nm by high-performance liquid chromatography by using a YMC-A312 column (YMC Co., Ltd.) and a mobile phase of 5% acetic acid-methanol (85:15; vol/vol) (42).

RESULTS

Susceptibility to quinolones and other antimicrobial agents. A total of 100 urinary isolates of *E. faecalis* collected from several hospitals throughout Japan in 1989 were tested for their quinolone susceptibilities. Although the susceptibilities to the fluoroquinolones were broad, about 10% of these strains were resistant to ≥25 μg of any fluoroquinolone per ml (Table 1). Among the fluoroquinolone-resistant isolates, two isolates, MS16968 and MS16996, were randomly selected for further studies. The susceptibilities of these two clinical isolates and ATCC 19433, a fluoroquinolone-susceptible strain, to quinolones and other antimicrobial agents are presented in Table 2. ATCC 19433 was intrinsically resistant

TABLE 1. Susceptibilities of 100 *E. faecalis* isolates to quinolone antimicrobial agents

Drug	MIC (μg/ml)[a]		
	Range	50%	90%
Norfloxacin	0.78–400	3.13	100
Ofloxacin	0.39–400	3.13	50
Ciprofloxacin	0.39–200	0.78	25
Temafloxacin	0.20–200	1.56	25

[a] MICs were determined by the agar dilution method. 50% and 90%, MIC for 50 and 90% of isolates, respectively.

to nalidixic acid, pipemidic acid, and a cephalosporin, ceftazidime, but was susceptible to fluoroquinolones, novobiocin, chloramphenicol, tetracycline, and ampicillin. On the other hand, MS16968 and MS16996 were highly and moderately resistant to fluoroquinolones, respectively. The MICs of the fluoroquinolones against MS16968 and MS16996 were 64- to 128-fold and 16- to 32-fold higher, respectively, than those for ATCC 19433. Although obvious cross-resistance was also observed among the fluoroquinolones, the susceptibilities of these two isolates to nonquinolone agents were almost equal to that of ATCC 19433.

Purification and properties of DNA gyrase. To determine whether the fluoroquinolone resistance in *E. faecalis* was due to an altered DNA gyrase, partially purified gyrases were prepared from ATCC 19433, MS16968, and MS16996 by affinity chromatography on novobiocin-Sepharose. Figure 1 shows the enzymatic properties of DNA gyrase from ATCC 19433. When fractions of the A and B subunits were assayed separately with relaxed pBR322 as a substrate (Fig. 1, lanes b and c), no DNA supercoiling activity was observable. Supercoiling activity was observed only when the two subunits were combined (Fig. 1, lane d). The specific activities of the purified subunits A and B were 5.7×10^3 and 1.0×10^4 U/mg of protein, respectively. The major protein bands in the A and B subunit preparations from ATCC 19433 were approximately 100,000 and 85,000 daltons, respectively (data not shown). These results indicate that the DNA gyrase of *E. faecalis* consists of two subunits, analogous to the DNA gyrases purified from *E. coli*, *P. aeruginosa*, *B. subtilis*, and *M. luteus* (39). We tested the supercoiling activities of intergeneric hybrids prepared from isolated gyrase subunits of *E. faecalis*, *B. subtilis*, and *E. coli*. An

TABLE 2. Susceptibilities of *E. faecalis* ATCC 19433, MS16968, and MS16996 to antibacterial agents

Drug	MIC (μg/ml) for *E. faecalis*[a]:		
	ATCC 19433	MS16968	MS16996
Norfloxacin	6.25	400	100
Ofloxacin	3.13	400	100
Ciprofloxacin	1.56	200	50
Temafloxacin	1.56	200	100
Nalidixic acid	>400	>400	>400
Pipemidic acid	400	>400	>400
Novobiocin	6.25	6.25	6.25
Chloramphenicol	6.25	12.5	12.5
Tetracycline	6.25	12.5	12.5
Ceftazidime	100	100	100
Ampicillin	3.13	3.13	3.13

[a] MICs were determined by the agar dilution method.

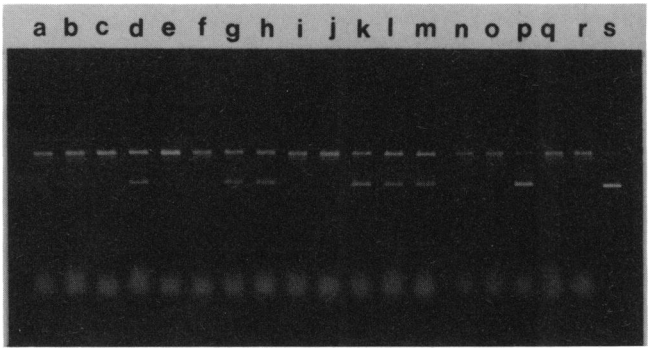

FIG. 1. Supercoiling activity of *E. faecalis* ATCC 19433 DNA gyrase. DNA gyrase reactions were performed as described in the text. Lane a, reaction mixture without enzyme (relaxed pBR322); lane b, subunit A (1 U); lane c, subunit B (1 U); lane d; subunit A plus subunit B (1 U each); lane e, ATP omitted; lane f, $MgCl_2$ omitted; lane g, 1.5 mM $MnCl_2$ added in place of $MgCl_2$; lane h, 1.5 mM $CaCl_2$ added in place of $MgCl_2$; lane i, subunit A from *B. subtilis* ATCC 6633; lane j, subunit B from *B. subtilis* ATCC 6633; lane k, subunit A from *B. subtilis* plus subunit B from *B. subtilis* (1 U each); lane l, subunit A from *E. faecalis* ATCC 19433 plus subunit B from *B. subtilis* (1 U each); lane m, subunit B from *E. faecalis* ATCC 19433 plus subunit A from *B. subtilis* (1 U each); lane n, subunit A from *E. coli* KL-16; lane o, subunit B from *E. coli* KL-16; lane p, subunit A from *E. coli* plus subunit B from *E. coli* (1 U each); lane q, subunit A from *E. faecalis* plus subunit B from *E. coli* (1 U each); lane r, subunit B from *E. faecalis* plus subunit A from *E. coli* (1 U each); lane s, supercoiled form of pBR322 (0.1 μg). Lanes e, f, g, and h are all contained 1 U each of A and B subunits from *E. faecalis* ATCC 19433.

active enzyme could be obtained by combining the A subunit of *E. faecalis* ATCC 19433 with the B subunit of *B. subtilis* ATCC 6633 (Fig. 1, lane l) and the subunit B of *E. faecalis* with the subunit A of *B. subtilis* (Fig. 1, lane m). This result provided evidence for the functional equivalence of *E. faecalis* and *B. subtilis* subunits. However, no interspecies complementation between *E. faecalis* subunits and *E. coli* subunits was observable with any combination of the subunits (Fig. 1, lanes q and r).

The *E. faecalis* gyrase was similar to the *E. coli*, *M. luteus*, or *B. subtilis* gyrase in its cofactor requirements. The supercoiling reaction absolutely required ATP and Mg^{2+} (Fig. 1, lanes e and f) at optimum concentrations of 3 and 1.5 mM, respectively (Fig. 2). A similar activity was obtained by the addition of Mn^{2+} or Ca^{2+} in place of Mg^{2+} (Fig. 1, lanes g and h; Fig. 2).

The A and B subunits from MS16968 and MS16996 were also separately purified by the same purification procedure, and their enzymatic properties were similar to those of the subunits from ATCC 19433.

Inhibition of DNA supercoiling activity. The inhibitory effects of ciprofloxacin on the DNA gyrases from ATCC 19433 and MS16968 are shown in Fig. 3. With the DNA gyrase from strain ATCC 19433, some inhibition by ciprofloxacin was seen at 25 to 50 μg/ml (Fig. 3, lanes e and f); nearly complete inhibition was seen at 100 μg/ml (Fig. 3, lane g). On the other hand, ciprofloxacin did not inhibit the supercoiling activity of MS16968 gyrase even at 800 μg/ml (Fig. 3, lane p). The IC_{50}s of fluoroquinolones, nalidixic acid, pipemidic acid, and novobiocin against the supercoiling activities of the DNA gyrase preparations from ATCC 19433, MS16968, and MS16996 were determined (Table 3). The IC_{50}s of norfloxacin, ofloxacin, ciprofloxacin, and tema-

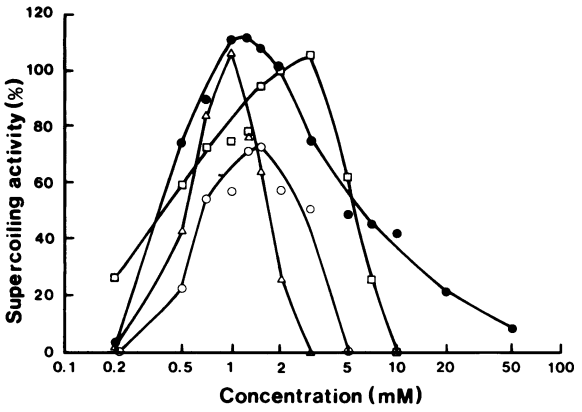

FIG. 2. ATP- and divalent cation-dependent DNA supercoiling activity of *E. faecalis* ATCC 19433 DNA gyrase. Supercoiling activity under the standard reaction conditions described in the text is the 100% reference. $MnCl_2$ and $CaCl_2$ were added in place of $MgCl_2$. Symbols: □, ATP; ●, $MgCl_2$; △, $MnCl_2$; ○, $CaCl_2$.

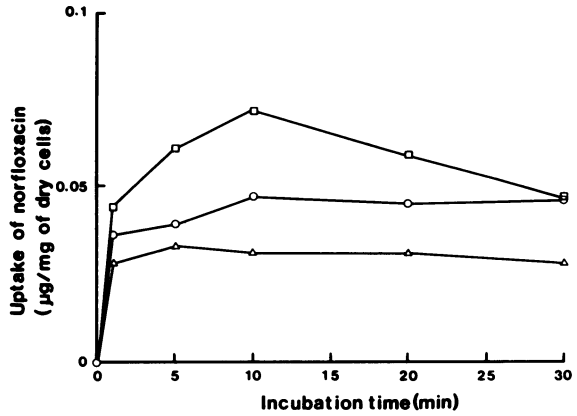

FIG. 4. Uptake of norfloxacin by *E. faecalis* ATCC 19433, MS16968, and MS16996. Details are given in the text. Symbols: ○, ATCC 19433; △, MS16968; □, MS16996.

floxacin against ATCC 19433 gyrase (As+Bs in Table 3) ranged from 23.8 to 74 μg/ml, whereas the supercoiling activities of the gyrase from MS16968 and MS16996 (Ar1+Br1 and Ar2+Br2, respectively, in Table 3) were unaffected, even at 800 μg of these drugs per ml. A difference in the level of resistance to fluoroquinolone inhibition of MS16968 and MS16996 gyrase could not be distinguished. IC_{50} assays were repeated with combinations of heterologous DNA gyrase preparations from ATCC 19433 and either MS16968 or MS16996. When the A subunit from ATCC 19433 was replaced by the A subunit from either MS16968 or MS16996 (Ar1+Bs or Ar2+Bs in Table 3), the supercoiling reaction was as resistant to the fluoroquinolones as the intact MS16968 or MS16996 holoenzyme was (Table 3). Conversely, reconstitution of the B subunit of either MS16968 or MS16996 gyrase with the A subunit from ATCC 19433 (As+Br1 or As+Br2 in Table 3) resulted in IC_{50}s comparable to those observed with the ATCC 19433 holoenzyme (As+Bs in Table 3). In contrast to the fluoroquinolones, but predictable from the MICs, the old quinolones nalidixic acid and pipemidic acid did not inhibit the supercoiling activity of any gyrase preparation. Novobiocin, a compound that competitively inhibits ATP binding to the B subunit (20), was equally active against all gyrase preparations (IC_{50}, 4 to 5 μg/ml).

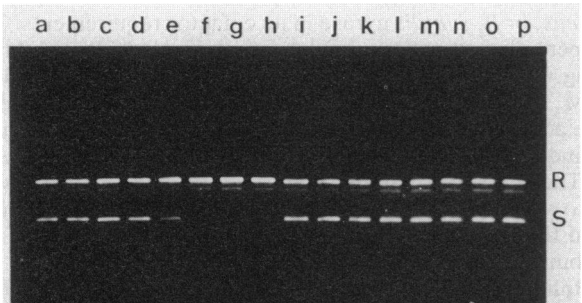

FIG. 3. Comparative inhibitory effects of ciprofloxacin on supercoiling activities of reconstituted subunit A and B proteins. The positions of relaxed (R) and supercoiled (S) pBR322 are indicated. Lane a, relaxed pBR322 with 1 U each of subunits A and B from ATCC 19433. Lanes b to g are the same as lane a, with ciprofloxacin at the following concentrations (in micrograms per milliliter): b, 3.13; c, 6.25; d, 12.5; e, 25; f, 50; g, 100; h, 200. Lane i, relaxed pBR322 with 1 U each of subunits A and B from MS16968. Lanes j to p are the same as lane i, with ciprofloxacin at the following concentrations (in micrograms per milliliter): j, 12.5; k, 25; l, 50; m, 100; n, 200; o, 400; p, 800.

Uptake of norfloxacin. The uptake of norfloxacin by ATCC 19433, MS16968, and MS16996 cells was compared (Fig. 4). No significant reduction in the amount of uptake was observed in MS16968 or MS16996 compared with that in ATCC 19433, indicating that a reduction in drug uptake does not account for the resistance to fluoroquinolones in *E. faecalis*. However, the uptake of norfloxacin by MS16968 was about twofold lower than that by MS16996. This result suggests that the difference in drug uptake contributes to the level of resistance, in addition to the DNA gyrase, because the MICs of fluoroquinolones for MS16968 were two- to fourfold higher than those for MS16996.

DISCUSSION

E. faecalis, which is known to be unique among streptococci for its resistance to a variety of antimicrobial agents, is generally regarded as being susceptible to penicillins, carbapenems, and recently developed fluoroquinolones such as ciprofloxacin and temafloxacin. In the present study, we documented the emergence of fluoroquinolone resistance among *E. faecalis* isolated from urinary tract infections. The mechanisms of fluoroquinolone resistance in gram-positive bacteria have not been fully elucidated, despite their clinical importance.

The *E. faecalis* DNA gyrase was partially purified by novobiocin-Sepharose affinity chromatography. This enzyme consisted of two components, subunits A and B, which had molecular weights similar to those of the *E. coli* gyrase subunits (40). Interspecies complementation experiments have shown that *E. faecalis* gyrase subunits could complement *B. subtilis* gyrase subunits, suggesting that subunits A and B of *E. faecalis* gyrase are the functional equivalents of the corresponding subunits of *B. subtilis* gyrase. Orr and Staudenbauer (33) reported that the B subunit of *B. subtilis* gyrase could complement the A subunit of *E. coli* gyrase, whereas no complementation was detected between the A subunit of *B. subtilis* gyrase and the B subunit of *E. coli*

TABLE 3. Susceptibility of the supercoiling reaction to DNA gyrase inhibitors

Drug	IC_{50} (μg/ml)[a]						
	As+Bs	Ar1+Br1	Ar2+Br2	As+Br1	As+Br2	Ar1+Bs	Ar2+Bs
Norfloxacin	74.0	>800	>800	ND[b]	ND	ND	ND
Ofloxacin	36.9	>800	>800	31.1	38.4	>800	>800
Ciprofloxacin	23.8	>800	>800	19.5	18.5	>800	>800
Temafloxacin	40.6	>800	>800	ND	ND	ND	ND
Nalidixic acid	>800	>800	>800	ND	ND	ND	ND
Pipemidic acid	>800	>800	>800	ND	ND	ND	ND
Novobiocin	4.7	4.2	4.5	4.7	4.5	ND	ND

[a] Calculated by measuring the supercoiled pBR322 DNA peak areas by densitometry. As, A subunit from ATCC 19433; Bs, B subunit from ATCC 19433; Ar1, A subunit from MS16968; Br1, B subunit from MS16968; Ar2, A subunit from MS16996; Br2, B subunit from MS16996; s, susceptible; r, resistant.
[b] ND, not determined.

gyrase. However, in this study, no complementation was observed between heterologous subunits of *E. faecalis* and *E. coli* gyrases. This suggests that the *E. faecalis* subunits can hardly interact efficiently with the *E. coli* subunits.

We also showed that the supercoiling reaction by the *E. faecalis* gyrase was inhibited by fluoroquinolones and novobiocin, but not by nalidixic acid or pipemidic acid. It has been reported that there is a poor correlation between IC_{50}s and MICs of fluoroquinolones for gram-positive bacteria such as *M. luteus* (10, 45) and *S. aureus* (13). However, in this study, the IC_{50}s of the fluoroquinolones correlated well with their respective MICs, even though the IC_{50} was about 10 to 20 times higher than its respective MIC. This correlation has been observed in gram-negative bacteria. The IC_{50}s of quinolones for the supercoiling activity of *E. coli* gyrase are generally one to two orders of magnitude greater than the corresponding MICs (7, 19, 24, 37). Hooper and Wolfson (19) suggested that low levels of inhibition of DNA supercoiling or inhibition of supercoiling within certain domains of DNA are critical in the antibacterial action of fluoroquinolones. This would help explain the discrepancy between the concentration of drug required to inhibit the supercoiling reaction and that required to inhibit bacterial growth.

To elucidate the mechanisms of resistance to fluoroquinolones in clinical isolates of *E. faecalis*, we purified DNA gyrase from two fluoroquinolone-resistant *E. faecalis* isolates, MS16968 and MS16996. The supercoiling activities of the DNA gyrases from both of the isolates were resistant to fluoroquinolones, but they were not resistant to novobiocin, an inhibitor that interferes with ATP binding to the B subunit of DNA gyrase. This suggests that the A subunit protein of each of the gyrase was altered. The gyrase A subunit from either of the resistant isolates conferred resistance to fluoroquinolones in heterologous gyrase subunit assays with the B subunit from the susceptible strain ATCC 19433. In contrast, the reconstituted gyrase consisting of subunit A from ATCC 19433 and subunit B from either of the resistant isolates retained sensitivity to fluoroquinolones. These results confirm that strains MS16968 and MS16996 carry alterations in the A subunit of DNA gyrase. However, these supercoiling assays did not explain the difference in the level of fluoroquinolone resistance between these two isolates.

We compared the uptakes of norfloxacin by these resistant isolates with the uptake by ATCC 19433 to determine whether other factors contributed to the fluoroquinolone resistance. Our results indicate that an alteration in the cell permeability to fluoroquinolones is not the major contributor to fluoroquinolone resistance. However, a difference in the amount of drug uptake may account for the difference in the level of fluoroquinolone resistance between these two isolates. In a recent report, Kojima et al. (26) demonstrated incomplete cross-resistance among fluoroquinolones with ciprofloxacin-resistant, methicillin-resistant *S. aureus* isolates and speculated that changes in permeability or in the efflux of fluoroquinolones contributed significantly to the fluoroquinolone resistance in these strains. The outer membrane of gram-negative cells has been widely accepted to be a barrier to the penetration of quinolones into the bacterial cell (16, 21, 22, 36). Since *S. aureus* does not have an outer membrane structure, Kojima et al. (26) also suggested that a barrier function might exist in the cytoplasmic membrane. Thus, it appears that the permeability or efflux of the drug may play a role in fluoroquinolone resistance in gram-positive bacteria. With regard to gram-negative bacteria, an energy-dependent system located at the bacterial inner membrane has been found to decrease norfloxacin uptake in *E. coli* (6). Further studies are needed to determine whether such a mechanism exits in gram-positive bacteria.

On the basis of these results, we conclude that one of the resistance mechanisms of *E. faecalis* against fluoroquinolones is an alteration in the DNA gyrase A subunit. Our study also shows the role of the DNA gyrase subunit A as the target for fluoroquinolones in *E. faecalis*. Recently, Hopewell et al. (23) have succeeded in cloning *S. aureus* genes which encode the DNA gyrase A and B proteins. From the DNA sequence analysis, a close homology between the *S. aureus* gyrase subunits and their counterparts in *B. subtilis* and *E. coli* was observed, including several conserved amino acid residues whose substitution in *E. coli* confers resistance to fluoroquinolones. The application of a similar genetic approach will enable the elucidation of the mechanisms of fluoroquinolone resistance in *E. faecalis* at the molecular level.

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