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Mechanisms of Clinical Resistance to Fluoroquinolones in *Staphylococcus aureus*

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Mechanisms of *Staphylococcus aureus* resistance to fluoroquinolones were characterized. Subunit A and B proteins of DNA gyrase were partially purified from fluoroquinolone-susceptible strain SA113 and resistant isolate MS16405, which was 250- to 1,000-fold less susceptible to fluoroquinolones such as ciprofloxacin, norfloxacin, ofloxacin, temafloxacin, and sparfloxacin than SA113 was. The supercoiling activity of the gyrase from SA113 was inhibited by the fluoroquinolones, and the 50% inhibitory concentrations of the drugs correlated well with their MICs. In contrast, the gyrase from MS16405 was insensitive to inhibition of supercoiling by all of the quinolones tested, even at 800 μg/ml. Combinations of heterologous gyrase subunits showed that subunit A from MS16405 conferred fluoroquinolone resistance, suggesting that an alteration in gyrase subunit A is a cause of the fluoroquinolone resistance in MS16405. Uptake of hydrophilic fluoroquinolones such as ciprofloxacin and norfloxacin by MS16405 was significantly lower than that by SA113. Furthermore, this difference was abolished by the addition of an energy inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone, suggesting that an alteration in an energy-dependent process, such as an active efflux of hydrophilic quinolones, may lead to decreased drug uptake and hence to increased resistance to fluoroquinolones in MS16405. These findings suggest that the fluoroquinolone resistance in MS16405 is due mainly to an alteration in subunit A of DNA gyrase and may also be associated with an alteration in the drug uptake process.

Infections caused by methicillin-resistant *Staphylococcus aureus* strains are a serious medical problem because only a few effective therapeutic agents are clinically available. Some fluoroquinolones, such as ofloxacin, ciprofloxacin and others, have been used for treatment of methicillin-resistant *S. aureus* infections. However, along with their greater use, the emergence of fluoroquinolone-resistant strains of *S. aureus* has been commonly reported (21, 24).

Studies with *Escherichia coli* and other gram-negative bacteria have shown that fluoroquinolones exert their antibacterial activities by inhibiting DNA gyrase, an essential bacterial topoisomerase II (27, 31, 32). The resistance of *E. coli* and *Pseudomonas aeruginosa* to fluoroquinolones has been shown to be associated with modification of DNA gyrase or alteration of outer membrane permeability (3, 7, 8, 10, 11, 13, 16, 22, 23). In regard to *S. aureus*, the exact role of DNA gyrase in fluoroquinolone action and resistance has not been fully explained, although its DNA gyrase has been partially purified by several workers (6, 28). Recently, Tanaka et al. (29) reported a close correlation of antibacterial activities of fluoroquinolones with their inhibitory activities against DNA gyrase of *S. aureus* FDA 209P and suggested that in *S. aureus*, as in gram-negative bacteria, DNA gyrase is a target enzyme of fluoroquinolones.

Hopewell et al. (12) succeeded in cloning *S. aureus* genes which encode the DNA gyrase A and B proteins. Their DNA sequence analysis revealed a close homology between the *S. aureus* gyrase subunits and their counterparts in *Bacillus subtilis* and *E. coli*, including several conserved amino acid residues whose substitutions in *E. coli* confer resistance to 4-quinolones. They have also suggested that amino acid substitutions in a region of the gyrase A protein are responsible for ciprofloxacin resistance in ciprofloxacin-resistant isolates of *S. aureus* (25). Thus, mutations in the DNA gyrase A subunit are implicated in fluoroquinolone resistance in *S. aureus*. However, enzymatic properties of the gyrase and its role in fluoroquinolone resistance in resistant clinical isolates have not been reported because of the difficulty in obtaining a sufficient quantity of purified gyrase (12, 30).

The biochemical approach to studying the mechanisms of fluoroquinolone resistance is to analyze the properties of gyrase proteins obtained from quinolone-resistant strains. In the present study, we partially purified DNA gyrase from a methicillin- and fluoroquinolone-resistant *S. aureus* isolate and analyzed its properties to elucidate the mechanisms of fluoroquinolone resistance. The possible contribution of fluoroquinolone uptake to resistance was also examined.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* SA113 (14), from the Laboratory of Drug Resistance in Bacteria, was used as a reference strain. A methicillin- and fluoroquinolone-resistant *S. aureus* strain, MS16405, was clinically isolated from sputum in 1988 and identified to the species level by the API Staph System (Bio Mérieux S.A., Marcy-l'Etoile, France). The strains were maintained at −70°C in 50% glycerol.

Drug susceptibility testing. MICs were determined by a

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twofold agar dilution method. One loopful (ca. 10^4 CFU) of a diluted culture broth was inoculated onto a modified Mueller-Hinton agar (Nissui Seiyaku Co., Ltd., Tokyo, Japan) plate. The MIC was defined as the lowest concentration of an antimicrobial agent that inhibited visible growth after 18 h at 37°C.

Chemical agents. Dithiothreitol, tRNA of *E. coli* W, bovine serum albumin, spermidine, phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, *p*-aminobenzamidine, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), lysostaphin, lysozyme, proteinase K, Brij 58, ATP, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co., St. Louis, Mo. Topoisomerase I was from Bethesda Research Laboratories, Geitherburg, Md. Plasmid pBR322 was purchased from Takara Shuzo Co., Ltd., Kyoto, Japan. Epoxy-activated Sepharose 6B was purchased from Pharmacia Fine Chemicals Co., Ltd., Tokyo, Japan. Subunits A and B of *B. subtilis* DNA gyrase were purified from strain ATCC 6633 by the procedure Orr and Staudenbauer (20). This *B. subtilis* gyrase had specific activities of 6.4×10^3 (subunit A) and 4.2×10^3 (subunit B) U/mg of protein, respectively. Subunits A and B of *E. coli* KL-16 DNA gyrase were purified as described previously (1, 23). This preparation of *E. coli* gyrase had specific activities of 1.7×10^4 (subunit A) and 1.0×10^4 (subunit B) U/mg of protein. The following antimicrobial agents were used: ciprofloxacin from Bayer Yakuhin Ltd., Tokyo, Japan; norfloxacin from Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan; ofloxacin from Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan; temafloxacin from Tanabe Seiyaku Co., Ltd., Osaka, Japan; sparfloxacin from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan; novobiocin, tetracycline, minocycline, and chloramphenicol from Sigma Chemical Co.; and methicillin from Banyu Pharmaceutical Co., Ltd., Tokyo, Japan. All other agents were obtained from their respective manufacturers.

Enzyme purification. *S. aureus* cells were grown in 10 liters of tryptic soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% glycine to the late log phase 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 dithiothreitol, 10% [vol/vol] glycerol) and 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; 150 ml of TGED buffer, 5 mM dithiothreitol–2 mM EDTA–2 mM EGTA–10 mM $MgCl_2$–75 mM KCl–1 mM phenylmethylsulfonyl fluoride–1 mM diisopropyl fluorophosphate–1 mM *p*-aminobenzamidine, 50 μg of lysostaphin per ml, and 1 mg of lysozyme per ml were successively added; and the cell suspension was incubated at 37°C for 1 h. Phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, *p*-aminobenzamidine, and EGTA were added to inactivate proteases and nucleases which would hamper further purification steps (12, 30). 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, Darmstadt, Germany) for 10 min, the extract was centrifuged at 135,000 × *g* for 90 min in a Beckman type 42.1 rotor. To the supernatant, 5% neutralized polyethylenimine was added drop by drop with stirring to a final concentration of 0.5% and stirring was continued for 30 min. After centrifugation at 12,000 × *g* for 15 min, the tubes were drained and the pellets were homogenized in 1 M NaCl in TGED buffer with a glass homogenizer. After being stirred for 30 min, the suspension was centrifuged and the supernatant was collected. 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 30 min, suspended in 50 ml of TGED buffer, and dialyzed against 3 liters of the same buffer for 12 h.

The dialysate was applied to a column of novobiocin-Sepharose 6B (bed volume, 20 ml) previously equilibrated with TGED buffer. Novobiocin-Sepharose was prepared by the procedure of Staudenbauer and Orr (26). 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). Subunit A eluted mainly with 2 M KCl in TGED buffer, and subunit B eluted with 2 M KCl–5 M urea in TGED buffer. In accordance with the nomenclature of the subunits of *E. coli* gyrase (26), the active fractions at 2 M KCl and 2 M KCl–5 M urea were designated subunits A and B, respectively, of *S. aureus* gyrase. Active fractions of subunits A and B were pooled separately, immediately dialyzed against 3 liters of TGED buffer for 12 h, 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 2 months. The protein assay was performed by the procedure of Bradford (2).

DNA supercoiling assay and IC_{50} determination. DNA gyrase supercoiling activity was assayed as described previously (5, 19). 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 (5). The standard reaction mixture (20 μl) contained 20 mM Tris hydrochloride (pH 7.5), 20 mM KCl, 2 mM $MgCl_2$, 2 mM spermidine, 1.5 mM ATP, 1 mM dithiothreitol, 10 μg of bovine serum albumin per ml, 30 μg of tRNA per ml, 0.1 μg of relaxed pBR322 which had been relaxed by topoisomerase I, a drug solution, and DNA gyrase subunits A and B (1 U of each). A control reaction with no drug was included. After incubation at 37°C for 1 h, the reaction was stopped by 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 Seisakusyo Co., Ltd, Kyoto, Japan). Densitometric tracings were used to determine the 50% inhibitory concentrations (IC_{50}s). The IC_{50} was defined as the concentration of a drug that inhibited 50% of the supercoiling activity of DNA gyrase in a standard reaction mixture (1, 19).

Uptake of quinolones by *S. aureus* cells. Fluoroquinolone uptake by intact cells was measured essentially as described previously (7, 19, 34). Cells were grown to an A_{570} of 0.7 at 37°C in antibiotic medium 3 (Difco Laboratories). The quinolone was added to the bacterial culture to a final concentration of 10 μg/ml. When needed, CCCP was added to the culture to a final concentration of 0.1 mM 10 min after addition of the drug. The amounts of cell-associated quinolones were measured by high-performance liquid chromatography with a YMC-312 column (YMC Co., Ltd., Kyoto, Japan). The mobile phases used were 5% acetic acid–methanol (80:20 [vol/vol]) for ciprofloxacin, norfloxacin, and ofloxacin and 5% acetic acid–methanol–acetonitrile (70:15:15 [vol/vol/vol]) for temafloxacin and sparfloxacin.

Determination of hydrophobicity of quinolones. Hydrophobicity of quinolones was determined essentially by the method of Hirai et al. (7). The concentrations of quinolones

TABLE 1. Susceptibilities of *S. aureus* SA113 and MS16405 to antimicrobial agents

Drug	MIC[a] (μg/ml) for *S. aureus*	
	SA113	MS16405
Ciprofloxacin	0.39	400
Norfloxacin	0.78	800
Ofloxacin	0.39	100
Temafloxacin	0.2	50
Sparfloxacin	0.05	12.5
Nalidixic acid	50	400
Novobiocin	0.39	0.39
Methicillin	1.56	200
Minocycline	0.10	50
Tetracycline	0.78	200
Chloramphenicol	6.25	100

[a] Determined by the agar dilution method.

in the aqueous phase were determined by high-performance liquid chromatography with a TSK-GEL ODS-80T_M column (Tosoh Co., Tokyo, Japan). The partition coefficient (*P*) was expressed as the ratio of the amount of the compound in the *n*-octanol phase to that in the aqueous phase.

RESULTS

Susceptibility to quinolones and other antimicrobial agents. A total of 212 clinical isolates of *S. aureus* collected from several hospitals throughout Japan between 1985 and 1989 were tested for quinolone susceptibility, and about 30% were resistant to ≥3.13 μg of ciprofloxacin per ml. Among the resistant isolates, *S. aureus* MS16405 was selected for its high resistance to fluoroquinolones. Susceptibilities of SA113, a fluoroquinolone-susceptible strain, and MS16405 to quinolones and other antimicrobial agents are shown in Table 1. SA113 is intrinsically resistant to nalidixic acid but is susceptible to the fluoroquinolones and other antimicrobial agents listed in Table 1. On the other hand, MS16405 showed high resistance to all of the quinolones tested; the MICs were 250- to 1,000-fold higher than those for SA113. This strain was also resistant to methicillin, tetracycline, minocycline, and chloramphenicol, while SA113 was susceptible to these antimicrobial agents. However, MS16405 was as susceptible to novobiocin as SA113 was.

Purification and properties of DNA gyrase. To determine whether the fluoroquinolone resistance of *S. aureus* was due to an altered DNA gyrase, partially purified gyrases were prepared from SA113 and MS16405 by affinity chromatography on novobiocin-Sepharose. Figure 1 shows the enzymatic properties of DNA gyrase from SA113. When fractions of subunits A and B were assayed separately (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 purified subunits A and B were 2.9×10^3 and 8.7×10^3 U/mg of protein, respectively. Interspecies complementation between gyrase subunits of *S. aureus* and *B. subtilis* was examined. An active enzyme could be obtained by combining subunit B of *S. aureus* gyrase with subunit A of *B. subtilis* gyrase (Fig. 1, lane m). However, the reciprocal combination of heterologous subunits (i.e., subunit A of *S. aureus* gyrase plus subunit B of *B. subtilis* gyrase) did not result in an active holoenzyme (Fig. 1, lane l). Furthermore, the combination of subunit B of *S. aureus* gyrase and subunit A of *E. coli* gyrase also showed weak supercoiling activity

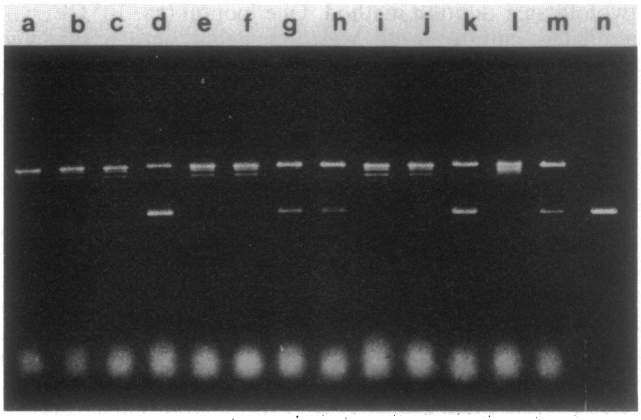

FIG. 1. Supercoiling activity of *S. aureus* SA113 DNA gyrase. DNA gyrase reactions were performed as described in the text. Lanes: a, reaction mixture without enzyme (relaxed pBR322); b, subunit A (1 U); c, subunit B (1 U); d, subunits A and B (1 U of each); e, ATP omitted; f, $MgCl_2$ omitted; g, 1.5 mM $MnCl_2$ added in place of $MgCl_2$; h, 1.5 mM $CaCl_2$ added in place of $MgCl_2$; i, subunit A from *B. subtilis* ATCC 6633; j, subunit B from *B. subtilis* ATCC 6633; k, subunit A from ATCC 6633 plus subunit B from ATCC 6633; l, subunit A from *S. aureus* SA113 plus subunit B from *B. subtilis* ATCC 6633; m, subunit B from *S. aureus* SA113 plus subunit A from *B. subtilis* ATCC 6633; n, supercoiled form of pBR322 (0.1 μg); e, f, g, and h, 1 U each of subunits A and B from *S. aureus* SA113.

(data not shown). These results indicate that the DNA gyrase of *S. aureus* consists of the two subunits, analogous to the DNA gyrase from other gram-positive bacteria, such as *B. subtilis* (20) and *Micrococcus luteus* (15), and provide evidence for the functional equivalence of the *S. aureus*, *B. subtilis*, and *E. coli* subunits.

S. aureus DNA gyrase was similar to *E. coli*, *B. subtilis*, or *M. luteus* gyrase in its cofactor requirements. The supercoiling reaction required ATP and Mg^{2+} (Fig. 1, lanes e and f) at optimum concentrations of 3 and 2 mM, respectively (data not shown). Slightly lower levels of activity were also obtained by addition of Mn^{2+} and Ca^{2+} in place of Mg^{2+} (Fig. 1, lanes g and h).

Subunits A and B from MS16405 were also separately purified by the same purification procedure, and their enzymatic properties were similar to those of the subunits from SA113.

Inhibition of DNA supercoiling activity. The inhibitory effects of ofloxacin on DNA gyrases from SA113 and MS16405 are shown in Fig. 2. With SA113 gyrase, some inhibition by ofloxacin was seen at 12.5 to 50 μg/ml (Fig. 2, lanes d to f); nearly complete inhibition was seen at 100 μg/ml (Fig. 2, lane g). On the other hand, MS16405 gyrase was hardly inhibited by ofloxacin, even at a concentration of 800 μg/ml (Fig. 2, lane p). The IC_{50}s of several fluoroquinolones, nalidixic acid, and novobiocin against the supercoiling activity of the gyrases from SA113 and MS16406 were determined (Table 2). The IC_{50}s of ciprofloxacin, norfloxacin, ofloxacin, temafloxacin, and sparfloxacin against SA113 gyrase (Table 2, As + Bs) ranged from 10.5 to 62 μg/ml, whereas the supercoiling activity of MS16405 gyrase (Table 2, Ar + Br) was unaffected, even at 800 μg of these drugs per ml. The older quinolone nalidixic acid did not inhibit the supercoiling activity of both gyrase preparations at 800 μg/ml. IC_{50} assays were repeated with combinations of heterologous gyrase preparations from SA113 and

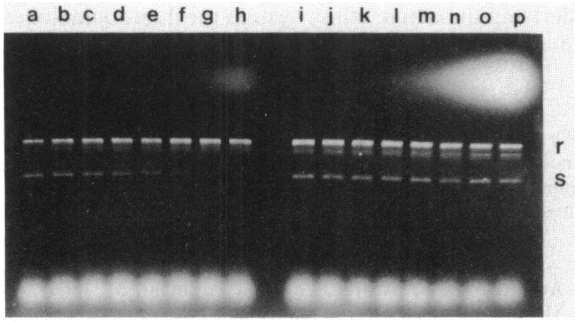

FIG. 2. Comparative inhibitory effects of ofloxacin on supercoiling activities of reconstituted subunit A and B proteins. The positions of relaxed (r) and supercoiled (s) pBR322 are indicated. Lane a contained relaxed pBR322 with 1 U each of subunits A and B from *S. aureus* SA113. Lanes b to h were the same as lane a, with ofloxacin 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 contained relaxed pBR322 with 1 U each of subunits A and B from MS16405. Lanes j to p were the same as lane i, with ofloxacin at the following concentrations (in micrograms per milliliter): j, 12.5; k, 25; l, 50; m, 100; n, 200; o, 400; p, 800.

MS16405. When subunit A from SA113 was replaced by subunit A from MS16405 (Table 2, Ar + Bs) the supercoiling reaction was as resistant to the fluoroquinolones as the intact MS16405 holoenzyme (Table 2). Conversely, reconstitution of subunit B of MS16405 gyrase with subunit A from SA113 (Table 2, As + Br) resulted in IC_{50}s comparable to those observed with the SA113 holoenzyme (Table 2, As + Bs). Novobiocin, an agent that competitively inhibits ATP binding to subunit B (9), was equally active against all gyrase preparations (IC_{50}, 0.4 to 0.7 μg/ml).

Uptake of fluoroquinolones. Fluoroquinolone uptake by SA113 and MS16405 cells was examined in the absence or presence of an energy inhibitor, CCCP (Table 3). Without CCCP, the levels of uptake by MS16405 were lower than those by SA113 with all of the drugs tested and the lowest amounts of uptake were observed with ciprofloxacin and norfloxacin. However, the level of sparfloxacin uptake by MS16405 was almost the same as that by SA113. In both strains, sparfloxacin uptake was higher than those of the other drugs, regardless of the absence or presence of CCCP, suggesting higher penetrability of *S. aureus* cells by sparfloxacin than other fluoroquinolones. By addition of CCCP, the levels of fluoroquinolone uptake significantly increased in both strains. This suggested either active efflux of the quinolone in *S. aureus* or active reduction of quinolone influx, as previously speculated by Yoshida et al. (34). Moreover, the difference in the levels of quinolone uptake between SA113 and MS16405 was almost abolished by addition of CCCP with all of these drugs. This finding indicated that an energy-dependent process, such as an active efflux, may play an important role in reducing drug uptake in MS16405.

TABLE 2. Susceptibility of the supercoiling reaction to DNA gyrase inhibitors

Drug	IC_{50} (μg/ml)[a]			
	As + Bs	Ar + Bs	As + Br	Ar + Br
Ciprofloxacin	19.4	>800	21.0	>800
Norfloxacin	62.0	>800	ND[b]	>800
Ofloxacin	16.5	>800	18.2	>800
Temafloxacin	15.3	>800	16.6	>800
Sparfloxacin	10.5	>800	9.2	>800
Nalidixic acid	>800	ND	ND	>800
Novobiocin	0.56	0.65	0.54	0.42

[a] Calculated by measuring the supercoiled pBR322 DNA peak areas by densitometry. Each value is the mean of two separate determinations. As, A subunit from SA113; Bs, B subunit form SA113; Ar, A subunit from MS16405; Br, B subunit from MS16405; s, susceptible; r, resistant.
[b] ND, not determined.

TABLE 3. Fluoroquinolone uptake by *S. aureus* cells

Drug	*P*[b]	Quinolone uptake (μg/mg of dry cells) at 20 min[a]			
		SA113		MS16405	
		Without CCCP	With CCCP[c]	Without CCCP	With CCCP
Ciprofloxacin	0.06	0.114	0.444	0.026	0.401
Norfloxacin	0.11	0.096	0.431	0.023	0.403
Ofloxacin	0.25	0.165	0.299	0.070	0.310
Temafloxacin	0.39	0.130	0.268	0.064	0.290
Sparfloxacin	0.70	0.494	0.697	0.368	0.733

[a] Each value is the mean of three separate determinations.
[b] Partition coefficient (*P*) in *n*-octanol–0.075 M phosphate buffer, pH 7.0.
[c] CCCP was added to the mixture at 0.1 mM 10 min after the addition of each quinolone.

DISCUSSION

The supercoiling reaction by partially purified DNA gyrase from fluoroquinolone-susceptible strain SA113 was inhibited by fluoroquinolones. The IC_{50}s of the fluoroquinolones tested correlated well with their respective MICs, with a correlation efficient of 0.917, although the IC_{50} of each drug was about 50- to 200-fold greater than its MIC. Similar results have recently been reported by Tanaka et al. (29) with the DNA gyrase purified from *S. aureus* FDA 209-P. These findings indicate that the differences in antibacterial activity between individual quinolones reflect variations in the ability to inhibit DNA supercoiling catalyzed by DNA gyrase. Thus, it appears that in *S. aureus*, as in gram-negative bacteria, the basis of fluoroquinolone activity is inhibition of DNA gyrase.

In this study, we described the mechanisms of fluoroquinolone resistance in a clinical isolate of *S. aureus* MS16405. The supercoiling activity of the gyrase from MS16405 was insensitive to fluoroquinolones but susceptible to novobiocin, suggesting that the subunit A protein was altered. Heterologous gyrase subunit assays confirmed that the decreased inhibition was conferred by resistant gyrase subunit A. Hence, we conclude that an alteration in subunit A of the gyrase is the major contributor to fluoroquinolone resistance in MS16405. This conclusion is consistent with recent genetic studies by Sreedharan et al. (25) on ciprofloxacin-resistant *S. aureus* isolates. Although they did not characterize the gyrase proteins, they suggested from the DNA sequence analysis of cloned gyrase genes that DNA gyrase subunit A mutations are responsible for the ciprofloxacin resistance of *S. aureus*.

To determine whether other factors contribute to the fluoroquinolone resistance of *S. aureus*, we analyzed the fluoroquinolone uptake of these strains. Our results suggest that the decreased uptake of the drugs is not the major contributor to resistance, because the amount of sparfloxacin taken up by MS16405 was virtually no different from

that taken up by SA113. However, we observed that uptake of hydrophilic quinolones, such as ciprofloxacin and norfloxacin, by MS16405 was significantly lower than their uptake by SA113. When deenergized by addition of CCCP, MS16405 accumulated over 15-fold more ciprofloxacin and norfloxacin, while SA113 accumulated only four- to fivefold more of these drugs. Consequently, the difference in uptake level between these strains was abolished. These results suggest the possibility that an alteration in the active efflux system decreased the uptake and hence increased the resistance levels in MS16405. Since the increase rate of sparfloxacin uptake in MS16405 caused by addition of CCCP was almost equal to that in SA113, the uptake of hydrophilic quinolones, but not that of hydrophobic quinolones, appears to be easily affected by an alteration in the efflux system. Thus, the decreased uptake of ciprofloxacin and norfloxacin may have been associated with the particularly high resistance of MS16405 to these drugs. Compared with ciprofloxacin and norfloxacin, ofloxacin and temafloxacin appear to be less affected by the alteration in the efflux system. This property may be explained by our finding that the hydrophobicities of these two drugs lie between those of ciprofloxacin or norfloxacin and sparfloxacin. In a previous report (34), Yoshida et al. described another type of quinolone-resistant isolate which exhibited high-level resistance to not only norfloxacin but sparfloxacin and a significant reduction in sparfloxacin uptake. This finding implies the presence of another efflux system which can excrete hydrophobic quinolones in *S. aureus*, or a further alteration in the efflux system for hydrophillic quinolones.

Recently, Yoshida et al. (33) reported that a *norA* gene which was cloned from a quinolone-resistant *S. aureus* strain (30) conferred relatively high resistance to hydrophilic quinolones, such as norfloxacin, enoxacin, ofloxacin, and ciprofloxacin, but only low or no resistance to hydrophobic quinolones, such as nalidixic acid and sparfloxacin, in *S. aureus*. They also showed that enoxacin uptake by *S. aureus* carrying the plasmid having the *norA* gene decreased by 50%, whereas the sparfloxacin uptake level was similar to that of the parent strain, SA113. Since the reduction of enoxacin uptake by the former strain was abolished by addition of CCCP, they speculated that the NorA polypeptide is a constituent of a membrane-associated active efflux pump of hydrophilic quinolones. Thus, it was implied that overproduction of the NorA protein is one of the fluoroquinolone resistance mechanisms in *S. aureus*. These findings also suggest that the active efflux system plays an important role in fluoroquinolone resistance in *S. aureus*.

Cohen et al. (4) reported the presence of an energy-dependent norfloxacin efflux pump in the inner membrane of quinolone-susceptible *E. coli* strains and suggested potential fluoroquinolone resistance mechanisms based on an increased gene dosage or expression of the carrier or an increased affinity of the efflux system for the fluoroquinolones. However, a mutation leading to such an alteration in a quinolone transport system in the cytoplasmic membrane has not been found. The presence of the drug efflux system implies the possibility of resistance mechanisms for nonquinolone antibacterial agents, such as those reported with class A and B tetracycline resistance in *E. coli* (18) and class L (TetL) tetracycline resistance in streptococci (17), which were found to be caused by energy-dependent decreased accumulation of the drug. However, we did not examine whether such resistance determinants are involved in the resistance to nonquinolone agents in MS16405. Further studies are important for elucidation of the mechanisms underlying the active efflux system for fluoroquinolones, its role in resistance, and its genetics in *S. aureus*.

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