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Inhibitory Effects of Ciprofloxacin and Sparfloxacin on DNA Gyrase Purified from Fluoroquinolone-Resistant Strains of Methicillin-Resistant *Staphylococcus aureus*

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The activities of new quinolones against 140 strains of methicillin-resistant *Staphylococcus aureus* were determined. From the relationship between the MICs of sparfloxacin and ciprofloxacin, fluoroquinolone-resistant *S. aureus* 6171 (MIC of sparfloxacin, 200 μg/ml; MIC of ciprofloxacin, 100 μg/ml) and fluoroquinolone-susceptible *S. aureus* FDA 209-P were selected for purification of the subunit A and B proteins of their DNA gyrases. The supercoiling activities of reconstituted A^rB^r (r, resistant) (from strain 6171) and A^rB^s (s, susceptible) gyrases were 40-fold more resistant to new quinolones than those of A^sB^s from FDA 209-P and A^sB^r gyrases. The 50% inhibitory doses of ciprofloxacin and sparfloxacin for A^mB^m (from mutant 19) and A^mB^s (m, moderately resistant) gyrases were 15- to 27-fold higher than those for A^sB^s and A^sB^m gyrases. These findings indicate that one of the resistance mechanisms of *S. aureus* against fluoroquinolones is a modification of the gyrase subunit A protein.

The new quinolones have potent antibacterial activities against gram-positive and gram-negative bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* (2, 5, 6, 8, 15, 19). However, resistance in *S. aureus*, especially in methicillin-resistant *S. aureus* (MRSA) isolates, and *P. aeruginosa* has increased, possibly owing to excessive or inappropriate use of new quinolones (12). It has been reported previously (1, 3, 10) that the mechanism of new quinolone resistance in clinical isolates of *P. aeruginosa* is a mutation in the A subunit of DNA gyrase and alteration in outer membrane permeability. Although the presence of an efflux mechanism in *S. aureus* has not yet been proved, it was recently inferred on the basis of increased quinolone accumulation by intact cells following treatment with energy inhibitors (21, 22). The inhibitory effects of quinolones on the DNA gyrase from fluoroquinolone-resistant *S. aureus* isolates were not examined, however, because of the difficulty encountered in the purification of DNA gyrase from these organisms. To this point, there have been no reports on the purification and the inhibitory properties of DNA gyrase from fluoroquinolone-resistant *S. aureus*.

In this report, we describe the purification and properties of subunit A and B proteins of DNA gyrase from a clinically isolated fluoroquinolone-resistant MRSA, *S. aureus* 6171.

MATERIALS AND METHODS

Bacterial strains and culture media. Fluoroquinolone-resistant *S. aureus* 6171 and quinolone-susceptible *S. aureus* FDA 209-P (20) were used in this study. Spontaneous ciprofloxacin-resistant mutants of FDA 209-P were isolated by the direct plating technique described below. Heart infusion broth (Nissui Seiyaku Co., Ltd.) was used to preculture the strains and to collect the cells.

Isolation of ciprofloxacin-resistant mutants of FDA 209-P. A single colony of strain FDA 209-P was inoculated into 10 ml of heart infusion broth, and aliquots of 0.1 ml of the overnight culture were plated (10^9 CFU) onto the surface of heart infusion agar (Nissui Seiyaku Co., Ltd.) plates containing 0.39 μg of ciprofloxacin per ml. Resistant colonies (single-step mutants) were counted after 48 h of incubation. The double-step (selective concentration of ciprofloxacin, 1.56 μg/ml) and triple-step (selective concentration of ciprofloxacin, 12.5 μg/ml) mutants were obtained in the same manner from among the single- and double-step mutants, respectively. Six representative colonies were selected from among the triple-step mutants, and the drug susceptibilities of these strains were tested with ofloxacin, ciprofloxacin, enoxacin, and sparfloxacin. From the MICs, one triple-step mutant (mutant 19) that was moderately resistant to both ciprofloxacin and sparfloxacin was chosen for further study.

Antibacterial agents and susceptibility. Ciprofloxacin (Bayer AG), sparfloxacin and enoxacin (Dainippon Seiyaku Co. Ltd.), ofloxacin (Daiichi Seiyaku Co., Ltd.), and novobiocin (Sigma Chemical Co.) were used. The MICs were determined by a twofold agar dilution method recommended by the Japan Society of Chemotherapy. One loopful (ca. 10^6 CFU) of a diluted culture broth was inoculated onto heart infusion agar plates. MICs were determined after incubation at 37°C for 18 h.

Isolation of DNA gyrase. *S. aureus* cells grown in heart infusion broth were collected by centrifugation at 10,000 × *g*, washed with TGED buffer (50 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol), and suspended in 100 ml of the same buffer; and 100 ml of glycerol (Wako) was added. Diisopropylfluorophosphate (final concentration, 1 mM; Sigma) was then added to the cell suspension. The cell suspension (ca. 20g) was frozen at −80°C for 2 h and was then thawed at 4°C; this process was repeated 10 times. All subsequent steps were performed at 0 to 4°C. After the cell suspension was sonicated 40 times for 15 s each time at output control 7 (model W-225R; Heat Systems-Ultrasonics, Inc.) in ice, 25 ml of 1 M KCl, 10 ml of 0.5 M EDTA, 0.5 ml of 1 M dithiothreitol, and 5 ml of 10% Brij 58 were added to the lysate (200 ml). The lysate was centrifuged at 6,000 × *g* for 20 min. For the removal of chromosomal DNA, a 50% solution of streptomycin sulfate prepared in water was added dropwise with stirring to a final

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concentration of 2%. After stirring for 30 min, the precipitate which formed was separated by centrifugation at 6,000 × *g* for 20 min, and the supernatant was collected by decantation. Then, solid ammonium sulfate was added to the supernatant to give 70% saturation (472 g/liter), and after stirring for 30 min, the precipitate was collected by centrifugation at 6,000 × *g* for 20 min and was dissolved in 50 ml of TGED buffer (pH 7.5). The solution was dialyzed three times against the same buffer at 4°C for a total of 12 h. The dialysate was applied to a novobiocin-Sepharose CL-6B column (17) which had been equilibrated in TGED buffer. The column was eluted stepwise with 0.2 M NaCl, 2 M NaCl, and 2 M NaCl–5 M urea in TGED buffer. The A-subunit protein was eluted with 0.2 M NaCl. The active A-subunit fraction was dialyzed four times against TGED buffer at 4°C for a total of 12 h, and the dialysate was further purified by using a heparin-Sepharose CL-6B (Pharmacia Fine Chemical Co. Ltd.) affinity column. Active subunit A fractions were eluted with 0.5 M NaCl in TGED buffer from the heparin column. The B-subunit protein was eluted with 2 M NaCl–5 M urea from the original novobiocin-Sepharose CL-6B column. The eluate fractions were immediately dialyzed four times against TGED buffer at 4°C for a total of 12 h, and the resulting dialysates of subunit A and B proteins were concentrated by dialyzing them against 50 mM Tris hydrochloride (pH 7.5)–50% glycerol–0.5 M EDTA–1 mM dithiothreitol for 4 h. The fractions were stable in storage at −80°C for several months. The protein assay was performed on the basis of the A_{280} (spectrophotometer UV-140-02; Shimadzu Co., Ltd.) and by the method of Lowry et al. (13).

DNA supercoiling assay and ID_{50} determinations. The supercoiling activity of DNA gyrase and 50% inhibitory doses (ID_{50}s) of quinolones were examined by using modifications of methods described in previous reports (18). One unit of gyrase, as defined by Gellert et al. (7), is that amount of enzyme which catalyzes the conversion of one-half of the relaxed closed-circular DNA to the supercoiled form in 2 h at 37°C in an agarose reaction mixture containing 0.15 μg of DNA. Gyrase subunit A activity was determined in the presence of excess gyrase subunit B. Gyrase subunit B activity was determined in the presence of excess gyrase subunit A. Plasmid pBR322 DNA, which was used as the substrate in the supercoiling assay of DNA gyrase, was relaxed by use of topoisomerase I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in a manner similar to that described previously (18). The reaction mixture (20 μl) contained 25 mM Tris hydrochloride (pH 8.0), 67 mM KCl, 5 mM $MgCl_2$, 1.25 mM spermidine hydrochloride, 1.7 mM ATP, 2 μg of *Escherichia coli* tRNA per ml, and 0.15 μg of relaxed pBR322 DNA. After approximately 1 U of each of gyrase subunits A and B was added to the reaction mixture, the mixture was incubated at 37°C for 2 h. The reaction was stopped by the addition of proteinase K (Sigma). The mixture was subjected to 1% agarose gel electrophoresis in TBE buffer (0.089 M Tris-borate and 0.002 M EDTA [pH 8.0]). Gyrase activities were determined following staining of the gel with ethidium bromide (5 μg/ml).

One unit of each of subunits A and B (final volume, 40 μl) was added to 15 μl of reaction mixture, and subsequently, a portion (5 μl) of the quinolones was added at various concentrations and the mixture was incubated at 37°C for 2 h. Quinolone solutions were prepared at the desired concentrations at the time of each experiment. The inhibitory effect on supercoiling was examined following gel staining. The gel was photographed with UV light. The negatives (positive/negative instant pack film type 665; Polaroid) were traced with a laser densitometer (LKB). The ID_{50} was calculated from the density of the band of supercoiled DNA.

TABLE 1. Susceptibilities of 139 *S. aureus* isolates to quinolone antimicrobial agents

Drug	MIC (μg/ml)[a]		
	Range	50%	90%
Ofloxacin	0.20–>100	12.5	100
Enoxacin	0.39–>100	50	>100
Ciprofloxacin	0.10–>100	25	>100
Sparfloxacin	<0.006–>100	3.13	25

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

Accumulation of sparfloxacin and ciprofloxacin by *S. aureus*. The accumulation of sparfloxacin and ciprofloxacin by intact cells was measured by the method of Hirai et al. (9), with slight modification. The bacteria were grown to the late logarithmic phase (A_{550} = 1.0) in antibiotic medium 3 (Difco), and sparfloxacin and ciprofloxacin were added to the bacterial culture at final concentrations of 10 μg/ml. The culture was incubated at 37°C with shaking. When needed, an energy inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma), was added to the culture to a final concentration of 100 μM 10 min after the addition of the quinolone. At various time intervals, 10 ml of the culture was rapidly chilled. The cells were collected by centrifugation, washed twice with 5 ml of 0.1 M phosphate buffer (pH 7.2), and resuspended in 1 ml of the same buffer. Lysostaphin (Sigma) was added to the suspension at a final concentration of 60 μg/ml. Each sample was allowed to stand at 37°C for 30 min and was then immersed in boiling water for 10 min; this was followed by centrifugation. The concentration of the quinolone in the supernatant was measured by bioassay by using *E. coli* NIH JC-2 (9).

RESULTS

Selection of ciprofloxacin-resistant mutants. Single-step mutants of *S. aureus* FDA 209-P arose at a frequency of 7.2×10^{-6} when the isolate was plated on selective medium containing 0.39 μg of ciprofloxacin per ml. However, single-step mutants could not be isolated at concentrations above 0.78 μg of ciprofloxacin per ml. Double-step mutants arose at frequencies of 6.4×10^{-5} and 7.7×10^{-7} when single-step mutants selected with 0.39 μg of ciprofloxacin per ml were plated onto selective media containing 1.56 and 3.13 μg of ciprofloxacin per ml, respectively. However, double-step mutants could not be isolated at concentrations above 6.25 μg of ciprofloxacin per ml. Triple-step mutants arose at frequencies of 2.3×10^{-5} and 7.1×10^{-9} when double-step mutants selected with 1.56 μg of ciprofloxacin per ml were plated onto selective media containing 6.25 and 12.5 μg of ciprofloxacin per ml, respectively. However, triple-step mutants could not be isolated at concentrations above 25 μg of ciprofloxacin per ml. MICs for all six mutants (triple-step mutants) indicated that they were moderately resistant to both ciprofloxacin and sparfloxacin. One of these mutants (mutant 19) was selected as a strain for purification of its DNA gyrase.

Susceptibilities to quinolones. A total of 139 MRSA strains were tested for their quinolone susceptibilities. The MICs for 50% and 90% of the 139 strains tested are given in Table 1, and the relationship between the susceptibilities of MRSA

TABLE 2. Relationship between susceptibilities of MRSA isolates to sparfloxacin and ciprofloxacin[a]

Sparfloxacin MIC (μg/ml)	No. of isolates with ciprofloxacin MIC (μg/ml) of:													
	0.012	0.025	0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	>100
>100													1	2	5
100														1	2
50															
25											1	1	1	1	8
12.5											1		3	7	18
6.25											4	2	5	2	
3.13										1	9	2	4	2	1
1.56											1		1	1	
0.78															
0.39							2		1	2					
0.20							1	1				3			
0.10					3	8	4	1	8	3	1				
0.05				1		2	7		3						
0.025					1										
0.012															

[a] A total of 139 isolates were tested.

isolates to sparfloxacin and ciprofloxacin are given in Table 2. The susceptibilities of 139 strains of MRSA to sparfloxacin and ciprofloxacin were similar to those reported previously (11) and were divided into two groups on the basis of their susceptibilities to sparfloxacin (Tables 1 and 2). Group 1 strains were moderately or highly resistant to hydrophilic quinolones but were susceptible to the hydrophobic quinolone (sparfloxacin), and group 2 strains were resistant to all of the fluoroquinolones, including the hydrophobic quinolone (sparfloxacin). From among the group 2 strains, we chose *S. aureus* 6171, which was highly resistant to both ciprofloxacin and sparfloxacin, for purification of its DNA gyrase.

The susceptibilities of *S. aureus* FDA 209-P, mutant 19, and 6171 to quinolones are given in Table 3. *S. aureus* FDA 209-P was susceptible, *S. aureus* mutant 19 was moderately resistant, and *S. aureus* 6171 was highly resistant to the quinolones.

Isolation of DNA gyrase. The elution patterns on the novobiocin-Sepharose CL-6B affinity column obtained with 0.2 M NaCl-, 2 M NaCl-, and 2 M NaCl–5 M urea-containing TGED buffers of *S. aureus* 6171 are given in Fig. 1. Similar elution patterns were also obtained with *S. aureus* FDA 209-P and mutant 19 (data not shown). The highest enzymatic activity of the subunit A protein was near the 0.2 M NaCl peak of A_{280}. Neither exonuclease nor restriction enzyme was found in these fractions. On the other hand, 2 M NaCl fractions had no enzymatic activity of subunit A protein, and we could remove many impurities such as exonuclease with TGED buffer containing 2 M NaCl. The highest enzymatic activity of the subunit B protein was also near the 2 M NaCl–5 M urea peak of A_{280}.

TABLE 3. Susceptibilities of *S. aureus* FDA 209-P, mutant 19, and 6171 to quinolones

Antimicrobial agent	MIC (μg/ml) for *S. aureus*:		
	FDA 209-P	Mutant 19	6171
Ofloxacin	0.39	25	200
Ciprofloxacin	0.20	12.5	100
Enoxacin	0.78	25	100
Sparfloxacin	0.10	12.5	200

Total protein concentrations and unit activity determinations are presented in Table 4. The A- and B-subunit preparations did not possess supercoiling activity when they were present alone in a gyrase reaction, but they did exhibit supercoiling activity when they were combined. The major protein bands in the A- and B-subunit preparations from FDA 209-P, mutant 19, and 6171 were approximately 85,000

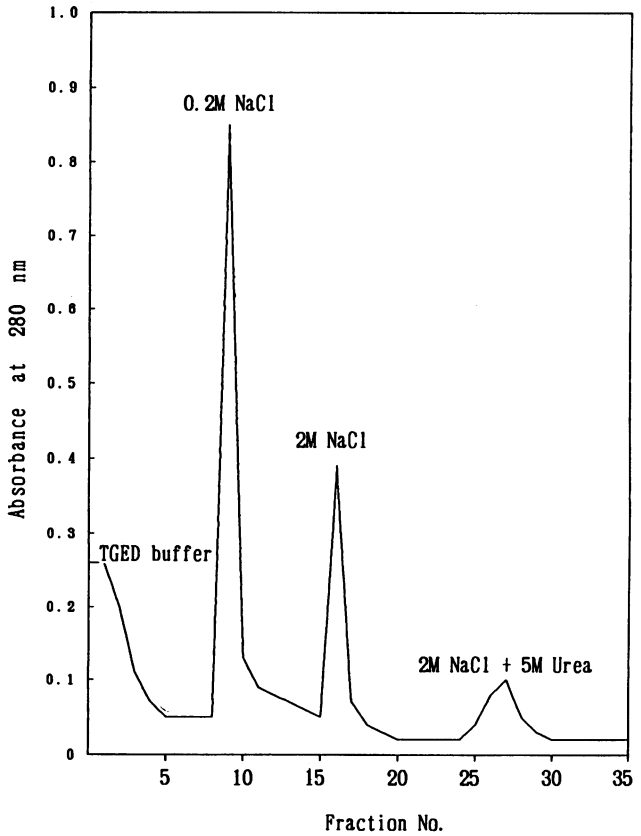

FIG. 1. Elution pattern of protein from *S. aureus* 6171 on a novobiocin-Sepharose CL-6B affinity column.

TABLE 4. Total protein concentrations and enzymatic activity of DNA gyrase subunits from *S. aureus*

Strain	Subunit	Total protein (mg)	Enzyme activity (U/μl)[a]	Sp act (U/mg of protein [10^3])	Total enzyme activity (U)
209-P FDA	A[b]	4.1	2.0	2.2	9.0×10^3
209-P FDA	B[c]	4.5	1.0	1.1	5.0×10^3
Mutant 19	A	2.0	0.7	1.3	2.6×10^3
Mutant 19	B	2.9	0.4	0.8	2.3×10^3
6171	A	1.4	0.5	0.7	1.0×10^3
6171	B	3.0	0.2	0.3	9.0×10^2

[a] One unit is defined as the amount of enzyme that results in one-half maximal supercoiling of the relaxed plasmid DNA substrate in a standard gyrase reaction.
[b] A, the A-subunit protein from the heparin column.
[c] B, the B-subunit protein from the novobiocin column.

and 80,000 Da, respectively (gel not shown). Scanning of the subunit lanes indicated that each subunit was at least 90% pure.

When the addition of ATP to the reaction mixture was omitted, there was no supercoiling activity for any of the three enzymes purified from FDA 209-P, mutant 19, and 6171. This result was similar to results of a recent study (20).

ID_{50} determinations. The supercoiling activities of reconstituted $A^s B^s$ (s, susceptible) and $A^r B^s$ (r, resistant) gyrases are shown in Fig. 2. Sparfloxacin inhibited the activity of $A^s B^s$ gyrase (Fig. 2B), and complete inhibition occurred at 50 μg/ml (Fig. 2B, lane c). In contrast, lanes b to d in Fig. 2A were the same as lane a in Fig. 2A, which contained sparfloxacin at high concentrations. The $A^r B^s$ gyrase was resistant to 800 μg of sparfloxacin per ml. The ID_{50}s of ciprofloxacin, sparfloxacin, and novobiocin against the supercoiling activities of reconstituted $A^r B^r$, $A^s B^s$, and $A^m B^m$ (m, moderately resistant) gyrases are given in Table 5. The activities of $A^r B^r$ and $A^r B^s$ gyrases indicated that they were highly resistant to ciprofloxacin and sparfloxacin (>800 μg/ml, respectively). The ID_{50}s of ciprofloxacin and sparfloxacin against $A^s B^s$, $A^s B^m$, and $A^s B^r$ gyrases ranged from 15 to 26 μg/ml. The activities of $A^m B^m$ and $A^m B^s$ gyrases indicated that they were moderately resistant to ciprofloxacin and sparfloxacin.

Accumulation of sparfloxacin and ciprofloxacin. The accumulation of sparfloxacin and ciprofloxacin by *S. aureus* FDA 209-P, mutant 19, and 6171 is given in Fig. 3. In *S. aureus*

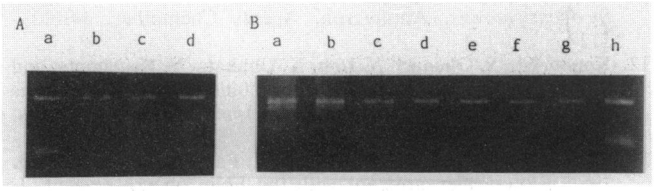

FIG. 2. Inhibition of supercoiling activity of reconstituted subunit A protein from *S. aureus* 6171 and subunit B protein from *S. aureus* FDA 209-P (A) and subunit A and B proteins from *S. aureus* FDA 209-P (B) by sparfloxacin. (A) Sparfloxacin concentrations (in micrograms per milliliter) were as follows: lane a, 0 (control); lane b, 800; lane c, 400; lane d, 200. (B) Sparfloxacin concentrations (in micrograms per milliliter) were as follows: lane a, 200; lane b, 100; lane c, 50; lane d, 25; lane e, 12.5; lane f, 6.25; lane g, 3.13; lane h, 0 (control).

TABLE 5. Inhibitory concentrations of quinolones and novobiocin on supercoiling activity of reconstituted DNA gyrase

Antimicrobial agent	ID_{50} (μg/ml) for reconstituted DNA gyrase of[a]:						
	$A^r B^r$	$A^r B^s$	$A^s B^r$	$A^s B^s$	$A^m B^m$	$A^m B^s$	$A^s B^m$
Ciprofloxacin	>800	>800	25	15	400	400	27
Sparfloxacin	>800	>800	26	20	390	400	26
Novobiocin	0.26	ND[b]	ND	0.18	ND	ND	ND

[a] The ID_{50} was calculated by measuring the supercoiled DNA peak by densitometry. A^r, A subunit from strain 6171; B^r, B subunit from strain 6171; A^s, A subunit from strain FDA 209-P; B^s, B subunit from strain FDA 209-P; A^m, A subunit from mutant 19; B^m, B subunit from mutant 19.
[b] ND, not determined.

FDA 209-P, sparfloxacin and ciprofloxacin were taken up into bacterial cells in the absence of CCCP. On the other hand, in mutant 19 and strain 6171, little sparfloxacin and ciprofloxacin was taken up in the absence of CCCP. However, this difference in quinolone accumulation was abolished by the addition of CCCP. These results suggest that the difference in the accumulation of sparfloxacin and ciprofloxacin observed among FDA 209-P, mutant 19, and 6171 contributes to the level of resistance, in addition to the observed changes in DNA gyrase.

DISCUSSION

By the method reported previously (18), we used lysostaphin for the lysis of staphylococcal cells. However, it appeared that commercially available lysostaphin contained some impurities such as DNases which may interfere with DNA gyrase activity. Therefore, in this study, we lysed bacterial cells by repeated freezing and thawing and then carrying out sonication to enhance the disruption of bacterial cells.

S. aureus possesses a potent protease (staphylococcal protease) (4, 14) which is not inhibited by EDTA. Although the activity of protease was suppressed by the addition of phenylmethylsulfonyl fluoride (1 mM) by the previously reported method (18), it was difficult to purify DNA gyrase proteins successfully because of the incompletion of the activity of phenylmethylsulfonyl fluoride as a protease inhibitor. In this study, we could purify DNA gyrase proteins stably, when we used diisopropylfluorophosphate as a more potent staphylococcal protease inhibitor.

Kojima et al. (11) reported that clinical isolates of ciprofloxacin-resistant MRSA are divided into two groups on the basis of their susceptibilities to sparfloxacin and norfloxacin; clinical isolates of group 1 are resistant to hydrophilic quinolones as a result of energy-dependent reduction in quinolone uptake, and clinical isolates of group 2 are resistant to all fluoroquinolones as a result of mutations of the *gyr* genes. We obtained similar results when we tested the susceptibilities of MRSA to new quinolones; clinical isolates were divided into two groups on the basis of their susceptibilities to sparfloxacin. From among the clinical isolates of group 2, *S. aureus* 6171, which potentially had quinolone resistance because of a mutation of the *gyr* gene, was selected for purification of its DNA gyrase. The supercoiling activity of the $A^r B^s$ gyrase in vitro was highly resistant to new quinolones. On the other hand, $A^s B^s$ and $A^s B^r$ gyrases were significantly more susceptible to these quinolones. On the basis of the results of these subunit switching experi-

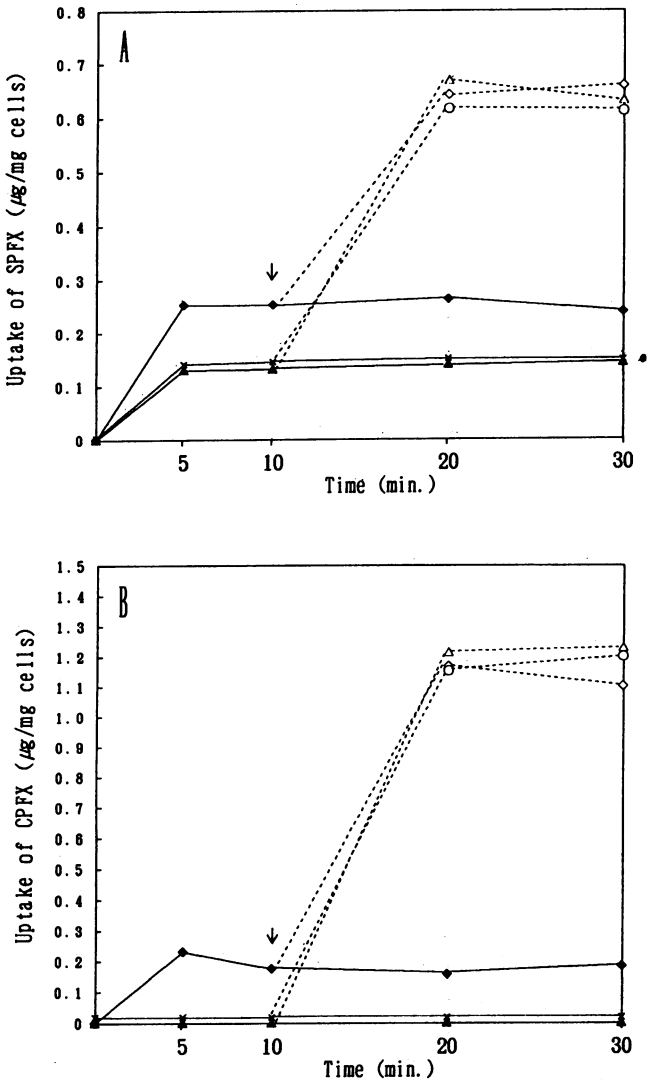

FIG. 3. Uptake of sparfloxacin (A) and ciprofloxacin (B) by *S. aureus* FDA 209-P, mutant 19, and 6171. Details are given in the text. Symbols: ◆, FDA 209-P without CCCP; ◇, FDA 209-P with CCCP; ▲, mutant 19 without CCCP; △, mutant 19 with CCCP; ×, 6171 without CCCP; ○, 6171 with CCCP. CCCP was added at the times indicated by arrows.

ments with purified A and B subunits from FDA 209-P and 6171, it could be suggested that one of the resistance mechanisms of *S. aureus* 6171 to quinolones is a modification of the subunit A protein.

Tanaka et al. (20) reported that the ID_{50}s of new quinolones against the DNA gyrase of *S. aureus* FDA 209-P were approximately 100-fold higher than the MICs. The ID_{50}s in this study were approximately 60- to 100-fold higher than the MICs, which agreed with the ID_{50}s reported by Tanaka et al. (20).

We also examined the accumulation of quinolones by *S. aureus* cells. *S. aureus* FDA 209-P cells took up more quinolones than did the resistant *S. aureus* cells in the absence of CCCP, but this difference in quinolone accumulation was abolished by CCCP. This result suggested that energy-dependent reduction systems in quinolone accumulation exist in *S. aureus* mutant 19 and 6171. This result was similar to that described in a recent report (22).

Although the MICs for *S. aureus* mutant 19 were about 100-fold higher than those for *S. aureus* FDA 209-P, the ID_{50}s on the DNA gyrase of *S. aureus* mutant 19 were only about 20-fold higher than those on the DNA gyrase of strain FDA 209-P. The high MICs for *S. aureus* mutant 19 may be due to the synergism between the low affinity of quinolones for the subunit A protein and energy-dependent reduction in quinolone uptake. The high MICs of new quinolones against *S. aureus* 6171 may also be due to the same synergistic mechanism. To confirm the mutation of the subunit A protein as a mechanism of quinolone resistance in *S. aureus* mutant 19 and 6171, further genetic approaches will be necessary. Therefore, genetic studies in *S. aureus* mutant 19 and 6171 are in progress (16).

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