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DNA Gyrase of *Staphylococcus aureus* and Inhibitory Effect of Quinolones on Its Activity

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DNA gyrase from *Staphylococcus aureus* FDA 209P was partially purified by lysostaphin treatment, saturation with ammonium sulfate, and affinity chromatography on heparin-Sepharose and with a concentrator (Centricon 30). It was found to consist of two subunits: α and β. The ability of new quinolone antibacterial agents such as norfloxacin, ofloxacin, and ciprofloxacin to inhibit DNA gyrase activity and cell growth was investigated. The inhibitory effects of the new quinolones against the activity of *S. aureus* DNA gyrase were in parallel with their antibacterial activities. The 50% inhibitory doses of norfloxacin, ofloxacin, and ciplofloxacin were 0.34, 0.31, and 0.24 μg/ml, respectively, while the 50% inhibitory doses of nalidixic acid and cinoxacin, which were less active against *S. aureus* FDA 209P, were 100 μg/ml or more.

The DNA gyrases are topoisomerases which catalyze the supercoiling of relaxed covalently closed circular DNA, which is known to be coupled with the hydrolysis of ATP. The enzyme from *Escherichia coli* (topoisomerase II) is composed of two different subunits, A and B, which are encoded by the genes *gyrA* and *gyrB*, respectively (5, 7). The *gyrA* protein constitutes the target for nalidixic acid and oxolinic acid, and the *gyrB* protein is the target for novobiocin and coumermycin A1 (6, 8, 9). The target of new quinolones, i.e., norfloxacin, ofloxacin, ciprofloxacin, and enoxacin, is subunit A; and these drugs exhibit marked inhibitory effects (18, 21). Evidence has been provided that other gram-negative bacteria possess DNA gyrase and that it is inhibited by quinolones (14, 19). In *E. coli* the active gyrase holoenzyme is a tetramer composed of two subunits, A2 and B2, which have molecular weights of 105,000 and 95,000, respectively (7). In regard to gram-positive bacteria, Orr and Staudenbauer (16) and Sugino and Bott (23) have reported the isolation of a gyrase from *Bacillus subtilis*, and Liu and Wang (12) have reported the isolation of a gyrase from *Micrococcus luteus*. Like *E. coli*, the *M. luteus* gyrase is a tetramer composed of two subunits, A and B, which have molecular weights of 115,000 and 87,000, respectively. The gyrases from these gram-positive bacteria catalyze the supercoiling of pBR322 DNA or ColE1 DNA derived from *E. coli* (24). New quinolone derivatives such as norfloxacin, enoxacin, ofloxacin, ciprofloxacin, and fleroxacin with a broad spectrum of antibacterial activity against gram-positive and gram-negative bacteria have been developed recently (1, 10, 11, 15, 20). These new quinolones are active against gram-positive bacteria, e.g., *Staphylococcus* spp., against which nalidixic acid scarcely has antibacterial activity. However, according to a recent report by Fu et al. (4) on the inhibitory effects of quinolones and other drugs on *M. luteus* DNA gyrase, both novobiocin and coumermycin A1 fully inhibit the supercoiling activity of the gyrase at low concentrations, near the MICs, but quinolones scarcely inhibit the gyrase. Furthermore, according to a report by Georgopapadakou and Dix (N. H. Georgopapadakou and B. A. Dix, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, A-33, p. 6) on the purification of *Staphylococcus aureus* ATCC 25923 DNA gyrase and inhibition by quinolones, the newer and potent quinolones norfloxacin, ciplofloxacin, and ofloxacin (MICs, <2.0 μg/ml) do not inhibit supercoiling (50% inhibitory concentrations, ca. >200 μg/ml). In this report, we describe the purification of DNA gyrase from *S. aureus* FDA 209P and the inhibitory effect of new quinolones on its activity.

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MATERIALS AND METHODS

Bacterial strain and culture media. *S. aureus* FDA 209P, which is preserved at the Department of Microbiology, Kyoto Pharmaceutical University, Kyoto, Japan, was used in this study. PYK broth was used to preculture the strain and to collect the cells. PYK broth was made up of the following: 5.0 g of polypeptone (Difco Laboratories, Detroit, Mich.), 5.0 g of yeast extract (Difco), 2.0 g of glucose (Wako Pure Chemical Industries Ltd.), and 3.0 g of K_2HPO_4 (Wako) per liter of distilled water.

Antibacterial agents and susceptibility. Norfloxacin (Kyorin Seiyaku Co., Ltd.), ofloxacin (Daiichi Seiyaku Co., Ltd.), ciprofloxacin (Bayer A.G.), nalidixic acid (Daiichi Seiyaku Co., Ltd.), cinoxacin (Sionogi Seiyaku Co., Ltd.), and pipemidic acid (Dainippon Seiyaku Co., Ltd.) were used. The MICs were determined by a twofold agar dilution method. One loopful (ca. 10^4 CFU) of a diluted culture broth was inoculated onto Mueller-Hinton agar (Nissui Seiyaku Co., Ltd.) plates. MICs were determined after incubation at 37°C for 18 h.

Partial purification of DNA gyrase from *S. aureus*. *S. aureus* FDA 209P cells grown in 20 liters of PYK broth were collected by centrifugation at 10,000 × *g*, washed with TGED buffer (50 mM Tris hydrochloride [pH 8.0], 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol), and stored at −80°C until use. The frozen cells (ca. 50 g) were thawed at 4°C; and 1.0 ml of 1.0 M dithiothreitol, 5.0 ml of 0.5 M EDTA, 7.5 ml of 1 M KCl, 2.5 ml of 10% Brij 58, and 0.1 ml of 1 M phenylmethylsulfonyl fluoride (Wako) were added. TGED buffer was added to make the final volume. Subsequently, 60 μg of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) per ml was added, and the mixture was incubated at 37°C for 30 min. All subsequent steps were performed at 0 to 4°C. After the sample was sonicated 10 times for 30 s at 50 W (TOMY SEIKO Co., Ltd.), the extract was

centrifuged at 100,000 × *g* on a rotor (55-2Ti; Beckman Instruments, Inc., Fullerton, Calif.) for 30 min. Solid ammonium sulfate was added to the supernatant to give 50% saturation (0.340 g/ml), and after stirring for 1 h, the precipitate was collected by centrifugation at 10,000 × *g* for 30 min and dissolved in 20 ml of TGED buffer (pH 8.0). The solution was dialyzed twice against the same buffer at 4°C for 6 h. The dialysate was applied to a heparin-Sepharose CL-6B (Pharmacia Fine Chemical Co., Ltd.) affinity column. The column was washed with 10 volumes of TGED buffer, and active fractions were eluted with TGED buffer containing 0.5 M NaCl or 5 M urea. The eluate fractions were immediately dialyzed twice against TGED buffer at 4°C for 6 h, and the dialysates were concentrated by using a concentrator (Centricon 30; Amicon Division, W. R. Grace & Co.). The fractions that eluted with 0.5 M NaCl and 5 M urea were added to 20 μl of the reaction mixture to measure the relaxation of pBR322 DNA and were regarded as subunits α and β fractions, respectively. Each enzyme fraction, to which 50% glycerol was added, was stored at −20°C and was used within 2 weeks. The protein assay was performed on the basis of the A_{280} (spectrophotometer UV-200S; Shimadzu Co., Ltd.) and by the method of Lowry et al. (13). The assay for contaminating endo- and exonucleases was as follows. A total of 0.5 μg of the relaxed pBR322 DNA was incubated in a reaction mixture (20 μl) either with or without (control) 5 μl of each fraction at 37°C for 2 h. After examination by agarose gel electrophoresis, the degree of digestion of relaxed pBR322 DNA was determined by using a laser densitometer (LKB Instruments, Inc., Rockville, Md.) to compare the quantity of undigested pBR322 DNA (control) with that treated with the α and β fractions.

Preparation of relaxed pBR322 DNA. The reaction mixture (150 μl) contained 50 mM Tris hydrochloride (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 15 μg of covalently closed circular pBR322 DNA, and 30 U of topoisomerase I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). One unit of topoisomerase I was defined as the amount of activity that relaxed 1 μg of supercoiled ϕX 174 phage DNA at 37°C for 30 min. After a 4-h incubation at 37°C, the reaction was stopped by the addition of an equal volume of phenol. After the phenol was removed with ether, the relaxed DNA was recovered from the aqueous phase by ethanol (1,500 μl) precipitation at −80°C for 6 h.

Supercoiling assay. The reaction mixture (20 μl) contained 20 mM Tris hydrochloride (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 2 mM spermidine hydrochloride, 2.0 μg of *E. coli* tRNA, 2 mM ATP, and 0.5 μg of relaxed pBR322 DNA. After a 5-μl portion of subunit α or β 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 0.8% agarose (Agarose 1600; Wako) gel electrophoresis in TBE buffer (0.1 M Tris borate and 1 mM EDTA [pH 8.0]). Gyrase activities were determined by staining the gel with ethidium bromide (0.5 μg/ml).

Inhibitory effects of quinolones. Portions of 5 μl of subunits α and β were added to 20 μl of reaction mixture, and subsequently, a portion (1 μl) of the quinolones at various concentrations was added 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 by gel staining. The gel was photographed with UV light. The negatives (Positive/Negative Instant Pack firm type 665; Polaroid) were traced with a laser densitometer (LKB). Each assay was performed twice for each quinolone examined and the 50% inhibitory dose (ID_{50}) was calculated from the volume of the closed circular DNA that was produced.

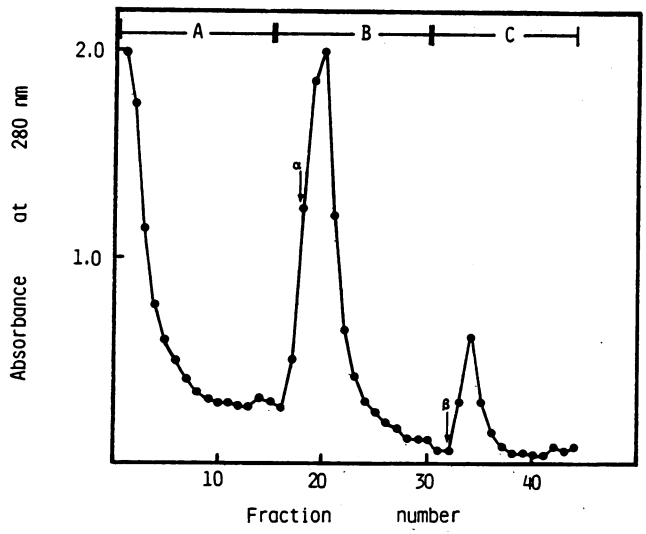

FIG. 1. Protein elution pattern on a heparin-Sepharose CL-6B affinity column. A, Fractions eluted with TGED buffer; B, fractions eluted with 0.5 M NaCl; C, fractions eluted with 5 M urea.

RESULTS

Partial purification of DNA gyrase from *S. aureus*. The elution patterns obtained with 0.5 M NaCl- and 5 M urea-containing TGED buffers are shown in Fig. 1. Gyrase activity was not correlated with the peak of A_{280}. Among the 0.5 M NaCl eluate fractions, the fraction showing the highest A_{280} value and the subsequently emerging fractions were exonuclease- or restriction enzyme-containing fractions. The same results were obtained when the column was developed on a linear gradient of 0.05 to 1.0 M NaCl (200 ml) (data not shown). When restriction enzyme contaminated the eluted fractions, linear pBR322 DNA appeared in the agarose gel (Fig. 2, lanes d to g). The protein content of 5 M urea fractions was generally lower compared with that after elution with 0.5 M NaCl. Neither exonuclease nor restriction enzyme was found in these fractions. Furthermore, the later 5 M urea fractions had no enzymatic activity against pBR322 DNA. Subunit α appeared in advance of the peak fraction of

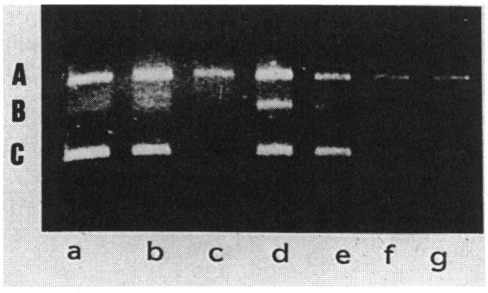

FIG. 2. Agarose gel electrophoresis of pBR322 DNA after the supercoiling assay was performed with selected eluate fractions. Lanes: a, B4 + C1; b, B4 + C2; c, B4 + C3; d, B5 + C1; e, B5 + C2; f, B5 + C3; g, B5 + C4. Fractions B and C are as defined in the legend to Fig. 1. The numbers indicate the fraction number. A, open circular DNA; B, linear DNA; C, covalently closed circular DNA.

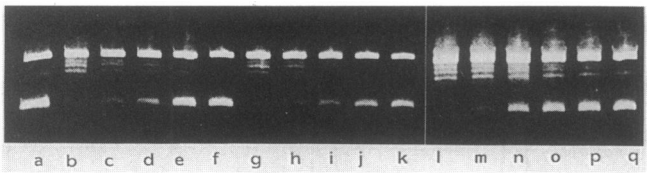

FIG. 3. Inhibitory effects of new quinolones against *S. aureus* FDA 209P gyrase. Lanes: a, no drug; b, ofloxacin, 1.61 μg/ml; c, ofloxacin, 0.81 μg/ml; d, ofloxacin, 0.40 μg/ml; e, ofloxacin, 0.20 μg/ml; f, ofloxacin, 0.10 μg/ml; g, norfloxacin, 3.22 μg/ml; h, norfloxacin, 1.61 μg/ml; i, norfloxacin, 0.81 μg/ml; j, norfloxacin, 0.40 μg/ml; k, norfloxacin, 0.20 μg/ml; l, ciprofloxacin, 0.81 μg/ml; m, ciprofloxacin, 0.40 μg/ml; n, ciprofloxacin, 0.20 μg/ml; o, ciprofloxacin, 0.10 μg/ml; p, ciprofloxacin, 0.05 μg/ml; q, ciprofloxacin, 0.025 μg/ml.

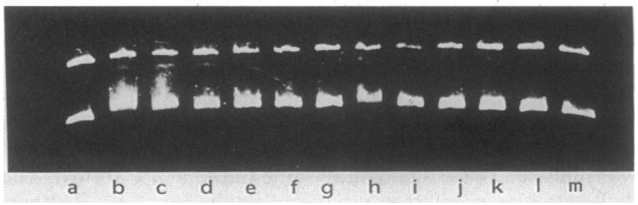

FIG. 4. Effects of nalidixic acid and cinoxacin against supercoiling of pBR322 DNA. Lanes: a, no drug; b, nalidixic acid, 103 μg/ml; c, nalidixic acid, 51.5 μg/ml; d, nalidixic acid, 25.7 μg/ml; e, nalidixic acid, 12.9 μg/ml; f, nalidixic acid, 6.4 μg/ml; g, nalidixic acid, 3.2 μg/ml; h, cinoxacin, 103 μg/ml; i, cinoxacin, 51.5 μg/ml; j, cinoxacin, 25.7 μg/ml; k, cinoxacin, 12.9 μg/ml; l, cinoxacin, 6.4 μg/ml; m, cinoxacin, 3.2 μg/ml.

A_{280}, and subunit β was in the first or second 5 M urea fraction (Fig. 2, lanes a to c). Fractions 18 and 32 had maximum activity (α and β subunits, respectively). Only when both subunits α and β (1 μg of protein per μl each) were added to the reaction mixture did gyrase activity appear. There was no activity in the other fractions. One unit of enzyme activity was defined as the amount that brought 50% of the relaxed pBR322 DNA to the supercoiled position in agarose gel electrophoresis, as described by Gellert et al. (7). The mixture containing both 5 μl of the subunit α and β fractions was regarded as 1 U of gyrase. However, subunit α or β alone had no enzymatic activity (data not shown).

Inhibitory effects of quinolones on *S. aureus* DNA gyrase. The inhibitory effects of quinolones were determined from gel electophoretograms. The supercoiling of the relaxed pBR322 DNA was inhibited by low concentrations of the new quinolones (Fig. 3). The ID_{50}s of norfloxacin, ofloxacin, and ciprofloxacin against *S. aureus* FDA 209P gyrase were 0.34, 0.32, and 0.21 μg/ml, respectively. These ID_{50}s were in parallel with the respective antibacterial activities given in Table 1. It was considered that these ID_{50}s were almost identical. On the other hand, the ID_{50}s of both nalidixic acid and cinoxacin were 100 μg/ml or more (Fig. 4), again corresponding to high MICs (Table 1).

DISCUSSION

DNA gyrase from *S. aureus* catalyzed the supercoiling of covalently closed circular DNA relaxed by topoisomerase I. The new quinolones inhibited this activity at concentrations near the MICs. Thus far, the purification of DNA gyrase consists of four steps: (i) lysis of bacteria, (ii) extractive separation by ultracentrifugation, (iii) saturation with ammonium sulfate, and (iv) column chromatography. For lysis of *S. aureus*, lysostaphin was used. Phenylmethylsulfonyl fluoride (1 mM) was added prior to lysostaphin treatment, because *S. aureus* possesses a potent protease (staphylococcal protease) (3, 17) which is not inhibited by EDTA. Sonication was carried out to enhance the disruption of cells in the viscous extract (DNA-lysed cell wall complex). In the purification of *B. subtilis* gyrase, 75% ammonium sulfate was added to the supernatant that was obtained after centrifugation at 12,000 rpm (24). In this study, however, 50% ammonium sulfate was used on the basis of an earlier report (16) that the molecular weight of *B. subtilis* gyrase is approximately 100,000 (subunit A, 115,000; subunit B, 87,000), which is comparable to that of *E. coli* DNA gyrase. The saturation in the purification of *E. coli* gyrase was performed at a concentration of 42% (19). In regard to column chromatography, Gellert et al. (7) isolated gyrase by using DEAE-Sepharose, hydroxyapatite, and valine-Sepharose successively. Orr and Staudenbauer (16, 22) used a novobiocin-Sepharose 6B affinity column for *B. subtilis* gyrase, in analogy with their purification of *E. coli* gyrase. We used the same novobiocin affinity column, but *S. aureus* gyrase was not obtained (data not shown). The reason for this is unknown. The β subunit of *S. aureus* FDA 209P gyrase may bind tightly to novobiocin in the affinity column, since this strain exhibited susceptibility to novobiocin (the MIC was below 0.05 μg/ml). Because heparin is a polyanion, it interacts with many cationic bioproducts, which is the basis of the purification with heparin-Sepharose CL-6B. When a heparin-Sepharose CL-6B column was used to purify *E. coli* gyrase, subunits A and B eluted together at a low salt concentration (19). However, it was assumed that subunit β of *S. aureus* gyrase would be eluted with 5 M urea with difficulty.

TABLE 1. Inhibitory effect of quinolones against *S. aureus* FDA 209P DNA gyrase

Quinolone	MIC (μg/ml)[a]	ID_{50} (μg/ml)[b]
Norfloxacin	0.39	0.34
Ofloxacin	0.39	0.31
Ciprofloxacin	0.39	0.24
Cinoxacin	>100	>100
Nalidixic acid	>100	>100

[a] Values are for 10^6 cells per ml.
[b] The ID_{50} was calculated by quantitatively measuring the supercoiled DNA peak in agarose gels by a densitometric assay.

The new quinolones exhibited potent inhibitory effects against *S. aureus* gyrase. Although this result differs from those of either the report of Fu et al. (6) or the report on inhibition of *M. luteus* DNA gyrase described by Zweerink and Edison (24), it seems reasonable to explain the antibacterial activities of the quinolones on the basis of the results obtained in the present study. The reason for the discrepancy between our results and those presented in previous reports (6, 24) is unknown. From our results, we found that the ID_{50}s were increased by contamination with another protein in the active fractions (data not shown). Gram-positive bacteria such as *S. aureus* possess a peptidoglycan layer which is not considered to provide a permeability barrier for substances. Thus, we considered that the discrepancy between the MIC and ID_{50}, like that found in *E. coli* (18), did not occur. The ID_{50}s of nalidixic acid and cinoxacin were 100 μg/ml or more. Reportedly, nalidixic acid exhibits a low affinity for *Pseudomonas aeruginosa* DNA gyrase (14). From a chemical structure viewpoint, nalidixic acid is a

naphthyridine with small C-7 substituents (—CH_3). The new quinolones are C-6-fluorinated quinolones, with piperazine at the C-7 position. In their study on structure-activity relationships of the new quinolone antibacterial agents with the target enzyme of *E. coli*, Domagala et al. (2) found that five- or six-membered rings, with or without small substituents at the C-7 position, have good gyrase-DNA complex-binding activities and have good to excellent MICs as well. Thus, neither nalidixic acid nor cinoxacin possesses even a little structural affinity for *S. aureus* gyrase.

In any event, the good antibacterial activities of the new quinolones against *S. aureus* are due to the potent inhibitory effects against the *S. aureus* gyrase. Further studies providing the details of why *S. aureus* gyrase shows such an intrinsic resistance to nalidixic acid or cinoxacin are needed.

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