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Differential Behaviors of *Staphylococcus aureus* and *Escherichia coli* Type II DNA Topoisomerases

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***Staphylococcus aureus gyrA* and *gyrB* genes encoding DNA gyrase subunits were cloned and coexpressed in *Escherichia coli* under the control of the T7 promoter–T7 RNA polymerase system, leading to soluble gyrase which was purified to homogeneity. Purified gyrase was catalytically indistinguishable from the gyrase purified from *S. aureus* and did not contain detectable amounts of topoisomerases from the *E. coli* host. Topoisomerase IV subunits GrlA and GrlB from *S. aureus* were also expressed in *E. coli* and were separately purified to apparent homogeneity. Topoisomerase IV, which was reconstituted by mixing equimolar amounts of GrlA and GrlB, had both ATP-dependent decatenation and DNA relaxation activities in vitro. This enzyme was more sensitive than gyrase to inhibition by typical fluoroquinolone antimicrobial agents such as ciprofloxacin or sparfloxacin, adding strong support to genetic studies which indicate that topoisomerase IV is the primary target of fluoroquinolones in *S. aureus*. The results obtained with ofloxacin suggest that this fluoroquinolone could also primarily target gyrase. No cleavable complex could be detected with *S. aureus* gyrase upon incubation with ciprofloxacin or sparfloxacin at concentrations which fully inhibit DNA supercoiling. This suggests that these drugs do not stabilize the open DNA-gyrase complex, at least under standard in vitro incubation conditions, but are more likely to interfere primarily with the DNA breakage step, contrary to what has been reported with *E. coli* gyrase. Both *S. aureus* gyrase-catalyzed DNA supercoiling and *S. aureus* topoisomerase IV-catalyzed decatenation were dramatically stimulated by potassium glutamate or aspartate (500- and 50-fold by 700 and 350 mM glutamate, respectively), whereas topoisomerase IV-dependent DNA relaxation was inhibited 3-fold by 350 mM glutamate. The relevance of the effect of dicarboxylic amino acids on the activities of type II topoisomerases is discussed with regard to the intracellular osmolite composition of *S. aureus*.**

DNA topoisomerases are ubiquitous enzymes responsible for controlling the topological state of DNA in cells (31, 33). They are charged with the task of resolving topological problems which arise during the various processes of DNA metabolism including transcription, recombination, replication, and chromosome partitioning during cell division. Topoisomerases can be divided into two main classes: type I enzymes, which cleave a single strand of DNA during the course of the reaction, and type II enzymes, which cleave both strands (32). The mechanism of these enzymes involves DNA cleavage and DNA strand passage through the break, followed by religation of the cleaved DNA. *Escherichia coli* has two type II topoisomerases, namely, DNA gyrase and topoisomerase IV, both of which are essential to the cell. Gyrase, through its unique ability to introduce negative supercoils into DNA (10), is involved during replication (mostly the initiation and elongation stages) and transcription in the maintenance of a critical superhelical density of DNA by relieving the positive superhelical tension generated ahead of the tracking complexes (31). Topoisomerase IV is also involved in supporting DNA replication, but despite obvious structural similarities with gyrase, topoisomerase IV appears to play a more specialized role in this process. The primary function of this enzyme is the decatenation of multiply linked daughter chromosomes during the terminal stages of DNA replication (1, 22, 36).

From a medical point of view, topoisomerases are important targets of a large variety of antitumor as well as antibacterial compounds (8). In *E. coli*, gyrase is the primary target of quinolones (9, 29), a widely used class of antibacterial agents (26). Topoisomerase IV is also sensitive to quinolones, but substantial evidence indicates that this enzyme is secondary to gyrase as a target of this family of drugs (5, 11, 14).

Although genes encoding homologs of *E. coli* gyrase and topoisomerase IV have been found in many microorganisms (12), biochemical studies have been carried out almost exclusively on the *E. coli* enzymes. *Staphylococcus aureus* gyrase, which is coded by two genes, *gyrA* and *gyrB*, has been purified to near homogeneity and has been partially characterized (18, 20, 30). *S. aureus* topoisomerase IV, coded by *grlA* and *grlB* (7), has not yet been purified. Genetic evidence strongly suggests that the primary target of fluoroquinolones in *S. aureus* is topoisomerase IV (6, 7, 34) and not gyrase, as it is in *E. coli* and *Neisseria gonorrhoeae* (2, 9, 29). This result is of prime importance owing to the rapid emergence and spread of fluoroquinolone resistance in *S. aureus* (25). A detailed understanding of the differences that are observed between type II topoisomerases from *E. coli* and *S. aureus* may open the way to the development of novel families of topoisomerase IV-targeted drugs showing no cross-resistance with fluoroquinolones. This study was therefore undertaken to characterize both gyrase and topoisomerase IV from *S. aureus* and to compare these two enzymes with each other as well as with their *E. coli* counterparts. This work provides biochemical evidence that

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TABLE 1. Nucleotide sequences of primers used in PCR

Primers	Nucleotide sequence[a]	Comments
2615 (forward)	gcg gga tcc atA TGG TGA CTG CAT TGT	Amplification of a 1.9-kb fragment containing *gyrB*
2613 (reverse)	TTC AAA gcT TCA GTT CAC AG	
4064 (forward)	gcc gAA gcT TTG AAG GAG GAA CTC TTG	Amplification of a 1.5-kb fragment containing the 5′ region of *gyrA*
2675 (reverse)	ggc gga att cGT TCT TCT GGA ATG AGA TCT	
3536 (forward)	GAT GAA GAT CTC ATT CCA GAA	Amplification of a 1.2-kb fragment containing the 3′ region of *gyrA*
3535 (reverse)	gga tCC TTA TTC TTC ATC ATC ATC TG	
3358 (forward)	ggc cgg atc cca taT GAG TGA AAT AAT TCA AGA TT	Amplification of a 1.4-kb fragment containing the 5′ region of *grlA* from fluoroquinolone-resistant strain 2C32C128B
3359 (reverse)	ggc cga gct cCA ATT CTT CTT TTA TGA CAT TC	

[a] The sequences of *S. aureus gyrA*, *gyrB*, and *grlA* are denoted in capital letters. Restriction enzyme sites are underlined.

topoisomerase IV is indeed the primary target of fluoroquinolones in *S. aureus*, and it sheds some light on significant differences in the catalytic properties of type II topoisomerases from *E. coli* and *S. aureus.*

MATERIALS AND METHODS

Materials. The pET11a expression vector and the host *E. coli* BL21(DE3) were from Novagen (28). T4 DNA ligase and restriction enzymes were from New England Biolabs, and the AmpliTaq DNA polymerase used for PCR amplification was from Perkin-Elmer. [4-^{14}C]sparfloxacin was from Dainippon Pharmaceuticals Co., Ltd., Osaka, Japan.

General methods. Oligonucleotide primers were synthesized with a 394 Applied Biosystems DNA Synthesizer from Perkin-Elmer. Protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Pierce) and with bovine serum albumin as a standard. Novobiocin-Sepharose was prepared by coupling novobiocin to epoxy-activated Sepharose CL-6B (Sigma) as described previously (27). *E. coli* DNA gyrase and topoisomerase IV were purified as described previously (7). Relaxed pBR322 DNA was prepared by incubating supercoiled pBR322 DNA with topoisomerase I purified from *E. coli* (15); this was followed by phenol and chloroform extraction and precipitation with ethanol.

Cloning and sequencing of *S. aureus* genes encoding DNA gyrase. DNA fragments containing the *gyrA* and *gyrB* genes were amplified from genomic DNA of *S. aureus* FDA 574 (7, 19) by PCR by using the primers listed in Table 1. A 1.9-kb DNA fragment was amplified at an annealing temperature of 35°C by using PCR primers 2613 and 2615. A 1.5-kb fragment and a 1.2-kb DNA fragment were amplified at the annealing temperature of 40°C by using primers 4064 and 2675 and primers 3536 and 3535, respectively. The DNA sequence of each PCR-amplified product, cloned into bacteriophage M13mp18 or M13mp19, was determined for at least three clones (24). Sequencing was performed either by the fluorescence-based dideoxy chain termination method, with sequencing reactions run on a 373A Applied Biosystems Sequencer (Perkin-Elmer), or by the dideoxy chain termination method with [α-^{35}S]dATP (24). The DNA sequence of each PCR-amplified product was found to be identical for at least three clones and was considered correct. The GyrA sequence determined in our laboratory differed from the published sequence (16) at the following positions: an isoleucine for a valine at position 598 (V598I), S668A, E709A, R837H, D862E, E877D, L884S, and E886D. Deletions of S826 and T827 were also found. The GyrB sequence was found to be identical to the sequence published by Brockbank and Barth (4) except at the following positions: L195I, D492H, T522I, and R599L.

Construction of *S. aureus* gyrase expression plasmid pXL2401. The two gyrase subunits were produced simultaneously by coexpressing *gyrA* and *gyrB* in *E. coli*. The 2.0-kb *Nde*I-*Bam*HI fragment containing the 3′-end coding sequence of *gyrA* was cloned into the pET11a vector. The resulting plasmid was digested with *Nde*I to introduce (i) the 2.0-kb *Nde*I-*Hin*dIII fragment containing *gyrB*, (ii) the 0.7-kb *Hin*dIII-*Nde*I fragment containing the intergenic sequence between *gyrB* and *gyrA*, and (iii) the 5′-end coding sequence of *gyrA* to form the expression plasmid pXL2401. This plasmid was used to transform *E. coli* BL21(DE3) cells. *E. coli* BL21(DE3)(pXL2401) was grown at 37°C in Luria-Bertani medium supplemented with 50 mg of ampicillin per liter to an optical density at 600 nm of 0.3, at which isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. Growth was continued at 30°C for 3 h, and the cells were harvested by centrifugation.

Construction of *S. aureus* GrlA^{Y80} expression plasmid pXL2742. A mutated GrlA^{Y80} subunit was produced in *E. coli* from the expression plasmid pXL2742. Use of primers 3358 and 3359 and genomic DNA of *S. aureus* 2C32C128B (6) allowed for the amplification of a 1.4-kb DNA fragment containing the point mutation in *grlA* corresponding to the serine to tyrosine substitution at position 80(Ser-80→Tyr substitution). A 0.75-kb *Nde*I-*Nde*I fragment from the amplified product was sequenced and cloned into the *Nde*I site of pXL2338 (7).

Purification of topoisomerases. Unless otherwise indicated, all steps were performed at 4°C.

Purification of *S. aureus* topoisomerase IV subunits expressed in *E. coli*. (i) Purification of GrlA. A cell extract was prepared from 5.0 g of wet cells of *E. coli* XL-1 Blue(pXL2340) as described previously (7), yielding 82 mg of protein. This crude extract was applied onto a MonoQ HR 10/10 column (Pharmacia) equilibrated with 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 1 mM dithiothreitol (DTT), and 10% (wt/vol) glycerol. Proteins were eluted at a flow rate of 3.0 ml/min with a 180-ml linear gradient of 0 to 0.6 M NaCl in the same buffer. Active fractions (5.7 mg of protein; 5 ml) were pooled and chromatographed on a gel permeation Superdex 200 HiLoad 26/60 column (Pharmacia) eluted at a flow rate of 1.5 ml/min with 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 5 mM DTT, and 250 mM NaCl. The GrlA fractions of the highest purity (1.1 mg) were pooled. GrlA^{Y80} was purified in a similar manner from *E. coli* XL-1 Blue(pXL2742).

(ii) Purification of GrlB. A cell extract was prepared from 5.0 g of wet cells of *E. coli* XL-1 Blue(pXL2320) as described previously (7). This extract (80 mg of protein) was applied onto a novobiocin-Sepharose CL-6B column (8 ml of gel) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 5 mM DTT, and 300 mM NaCl. After washing the column with the same buffer, GrlB was eluted at a flow rate of 0.8 ml/min with 10 ml of 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 5 mM DTT, 2 M NaCl, and 5 M urea. Urea was removed by passing the sample through a Sephadex G-25 column (50 ml of gel) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 5 mM DTT, 100 mM NaCl, and 10% (wt/vol) sucrose. The protein fraction (12 mg; 12 ml) was chromatographed in 4-ml aliquots on a gel permeation Superdex 200 HiLoad 26/60 column (Pharmacia) eluted at a flow rate of 1.5 ml/min with 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 5 mM DTT, and 250 mM NaCl. The GrlB fractions of the highest purity (1.9 mg) were pooled.

Purification of DNA gyrase from *S. aureus.* Cells of *S. aureus* FDA 209-P were grown in 20 liters of tryptic soy broth (Difco Laboratories) and lysed as described previously (18). After a brief sonication to reduce viscosity, the lysate (3.8 g of protein) was subjected to streptomycin sulfate precipitation and then ammonium sulfate fractionation as reported previously (20). The ammonium sulfate precipitate was dissolved in 125 ml of TGED buffer (50 mM Tris-HCl [pH 7.7], 10% glycerol, 1 mM EDTA, 1 mM DTT) and was chromatographed through a column of 500 ml of Sephadex G-25 eluted with TGED buffer. The protein fraction (2.45 g; 125 ml) was collected and applied at 12°C to a novobiocin-Sepharose CL-6B column (8 ml of gel) equilibrated with TGED buffer. After washing the column with 0.2 M sodium chloride in TGED buffer, the gyrase was eluted with 7.5 ml of TGED buffer containing 2 M sodium chloride and 5 M urea. Urea was removed by gel permeation through PD-10 columns (Pharmacia) equilibrated with TGED buffer. After this step gyrase was about 25% pure, as revealed by Coomassie staining after polyacrylamide gel electrophoresis (total amount of protein, 2.3 mg). In some instances, this gyrase preparation was further chromatographed through a Superdex 200 HR 10/30 column (Pharmacia) equilibrated and eluted at 8°C with TGED buffer containing 600 mM potassium glutamate, yielding a preparation that was 75% pure.

Purification of *S. aureus* DNA gyrase expressed in *E. coli.* *E. coli* BL21(DE3)(pXL2401) cells obtained from 3 liters of culture were resuspended in 25 ml of 100 mM Tris-HCl (pH 8.0) containing 10% glycerol, 75 mM KCl, 1 mM EDTA, 1 mM benzamidine, 1 mM Pefabloc SC [4-(2-aminoethyl)benzene-

sulfonyl fluoride hydrochloride; Merck], and 1 mg of lysozyme per ml. The suspension was incubated for 30 min at 20°C and was then centrifuged at 50,000 × *g* for 1 h. The gyrase was purified from the supernatant by a two-step chromatographic procedure on novobiocin-Sepharose CL-6B and then gel permeation on Superdex 200 HR 10/30, as described above with gyrase extracted from *S. aureus* FDA 209-P, yielding 600 μg of pure enzyme.

Topoisomerase reactions. Unless indicated otherwise, topoisomerase reactions were carried out as described below and DNA quantification in agarose gels was done by scanning densitometry after electrophoresis and ethidium bromide staining as described previously (11). IC_{50}s (mean of two independent determinations) were defined as the concentration of drug (in micrograms per milliliter) required for a 50% reduction of enzymatic activity under these conditions.

(i) Decatenation of kDNA. Standard reaction mixtures (40 μl) containing 50 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 5 mM DTT, 1.5 mM ATP, 50 μg of bovine serum albumin per ml, 350 mM potassium glutamate, 0.9 μg of kinetoplast DNA (kDNA; from *Crithidia fasciculata*; TopoGen, Inc.), and appropriate amounts of GrlA and GrlB (to give 1 U of decatenation activity) were incubated at 37°C for 1 h. The reactions were terminated, and the mixtures were processed and analyzed as described previously (21). One unit of decatenation activity was defined as the amount of topoisomerase IV required to fully decatenate 50% of the kDNA present in the reaction mixture in 1 h under the conditions described above. Fully active GrlAB protein was reconstituted by preincubating GrlA and GrlB at 4°C for 30 min. In the course of protein purification, GrlA and GrlB were detected by their ability to complement their partner protein (GrlB and GrlA, respectively) in the decatenation test. *E. coli* topoisomerase IV-dependent activity was assayed similarly at a concentration of 170 mM potassium glutamate.

(ii) Negative supercoiling of relaxed pBR322 plasmid DNA. Standard reaction mixtures (40 μl) containing 50 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 5 mM DTT, 1.5 mM ATP, 50 μg of bovine serum albumin per ml, 20 mM potassium chloride, 700 mM potassium glutamate, 0.4 μg of relaxed pBR322 DNA, and *S. aureus* gyrase were incubated at 37°C for 1 h. The reactions were terminated by the addition of 7 μl of 50 mM EDTA, 30% (wt/vol) sucrose, 1% sodium dodecyl sulfate (SDS), and 0.1% bromophenol blue and were analyzed by electrophoresis through a 1% agarose gel in 90 mM Tris borate–2 mM EDTA (pH 8.3). One unit of supercoiling activity was defined as the amount of gyrase required to catalyze the supercoiling of 50% of the relaxed pBR322 DNA present in the reaction mixture in 1 h under the conditions described above.

(iii) Relaxation of negatively supercoiled pBR322 plasmid DNA. Relaxation activities were assayed essentially as described previously (21) in 40 μl of 50 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 5 mM DTT, 1.5 mM ATP, 5 mM spermidine, 50 μg of bovine serum albumin per ml, 20 mM potassium chloride, and 0.6 μg of negatively supercoiled pBR322 DNA as the substrate. One unit of relaxation activity was defined as the amount of enzyme required to relax 50% of the supercoiled pBR322 DNA in the reaction mixture in 30 min at 37°C.

(iv) Topoisomerase-mediated cleavage with linear DNA. Cleavage reactions were carried out in 25 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 0.5 mM DTT, 3 μg of bovine serum albumin per ml, 10 mM $MgCl_2$, 120 mM KCl, 10 mM ATP, 10,000 dpm of 3′-end-labeled linearized pBR322 DNA (23), topoisomerase (150 ng of *E. coli* gyrase or 240 ng of *S. aureus* gyrase or 750 ng of *E. coli* topoisomerase IV or 140 ng of *S. aureus* topoisomerase IV), and drug in a final volume of 20 μl. In experiments with *S. aureus* gyrase, 0.7 M potassium glutamate (KGlu) replaced KCl. The reactions were performed at 37°C for 1 h and were terminated by the addition of 5 μl of 2.5% SDS–2.5 mg of proteinase K per ml. After 30 min at 37°C, 5 μl of 30% glycerol–1% SDS–50 mM EDTA–0.05% bromophenol blue was added and samples were analyzed by electrophoresis through 1% agarose gels and autoradiographed. Studies of inhibition by drugs were performed with 2.2 U of gyrase or topoisomerase IV.

(v) Topoisomerase-mediated cleavage with supercoiled DNA. The double-stranded DNA breaks generated upon incubation of topoisomerase IV or gyrase with 100 μg of ciprofloxacin per ml under the conditions used to assay decatenation and supercoiling activities, respectively, were monitored by measuring the conversion of negatively supercoiled pBR322 plasmid DNA to linear molecules. After a 30-min incubation at 37°C, the reactions were stopped by the addition of SDS-proteinase K as described above and the various DNA species were quantified by scanning densitometry after separation on 1% agarose gels.

Binding experiments. The reactions were performed in 50 μl of cleavage buffer (see above) containing 2 μg of negatively supercoiled pBR322 DNA, [4-^{14}C]sparfloxacin (0.85 mM; 460 MBq/mmol), and 8 μg (20 pmol) of purified gyrase. After incubation for 1 h at 30°C, the mixtures were applied to select-D G-50 spin columns (5 Prime-3 Prime Inc.) and were processed as described by the manufacturer. The amount of [4-^{14}C]sparfloxacin bound to the macromolecules was determined by liquid scintillation counting.

RESULTS

Purification of *S. aureus* gyrase. Gyrase was initially purified from *S. aureus* FDA 209-P by a procedure similar to that described previously (18, 20), yielding a preparation showing two major protein bands with M_rs of approximately 70,000 and 100,000, respectively, after electrophoresis under denaturing conditions. This preparation also showed several protein bands with M_rs in the range of 60,000 to 100,000. Two of these bands could well correspond to the A and B subunits of DNA topoisomerase IV, an enzyme recently identified in *S. aureus*. Although functionally different, gyrase and topoisomerase IV present obvious structural similarities, so the possibility that the two enzymes had copurified could not be excluded. For this reason, our gyrase preparation was not considered satisfactory as a starting material for subsequent enzymological studies, and the production of *S. aureus* gyrase in *E. coli* was undertaken. The *gyrA* and *gyrB* genes from *S. aureus* FDA 574 were cloned into pXL2401, a T7 promoter-based expression vector, and were coexpressed in *E. coli* BL21(DE3). This resulted in the synthesis of soluble gyrase to a level of about 1 to 2% of total cellular protein. Gyrase was purified to homogeneity from 3 liters of induced culture. SDS-polyacrylamide gel analysis of the purified gyrase (Fig. 1) revealed two bands with apparent molecular weights of about 105,000 and 75,000, corresponding to GyrA and GyrB, respectively. Microsequencing of this preparation gave two N-terminal sequences, A?LPQSRIN (GyrA) and VTA?SDVNN (GyrB). By analogy with the structure of *E. coli* gyrase, which is a heterotetramer, $GyrA_2GyrB_2$, the *S. aureus* GyrA and GyrB polypeptides were expected to be present in equimolar amounts in the purified protein. Quantitative data obtained during sequencing indicated a slight molar excess of GyrA over GyrB (molar ratio, 3/2) in pure gyrase purified from *S. aureus*. The significance of this result was not investigated. Coomassie blue staining after electrophoresis seemed to indicate an even greater excess of GyrA over GyrB (Fig. 1, lane 2), probably because GyrA stains better than GyrB under our experimental conditions.

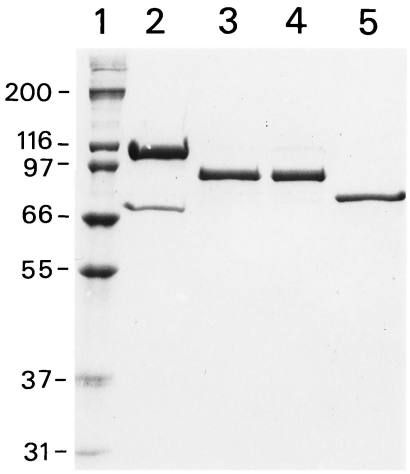

FIG. 1. SDS-polyacrylamide gel analysis of gyrase and topoisomerase IV subunits of *S. aureus* after purification. Molecular mass markers (lane 1, with sizes [in kilodaltons] indicated on the left), gyrase [lane 2; 2.0 μg purified from *E. coli* BL21(DE3)(pXL2401)], GrlA (lane 3; 1.7 μg), $GrlA^{Y80}$ (lane 4; 1.2 μg), and GrlB (lane 5; 1.3 μg) were electrophoresed through a 10% polyacrylamide gel containing SDS and were stained with Coomassie blue.

Characterization of *S. aureus* gyrase purified from *E. coli*. The supercoiling activities of *S. aureus* gyrase reported in the literature are extremely low (18, 20), i.e., about 3 orders of magnitude lower than that of *E. coli* gyrase. By modifying the incubation conditions, we found that KGlu could dramatically enhance *S. aureus* gyrase activity, from 1 U/μg of enzyme in the absence of KGlu to 500 U/μg in the presence of 700 mM KGlu, reaching supercoiling activities similar to that of the *E. coli* enzyme. At a concentration of 700 mM, the potassium salts of

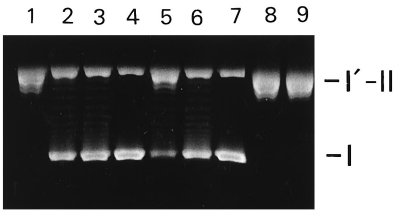

FIG. 2. Comparison of supercoiling activities of *S. aureus* gyrase purified from strain FDA 209-P and from *E. coli*. Standard reaction mixtures containing either no enzyme (lane 1) or 12.5, 18.75, 25, and 25 ng of *S. aureus* gyrase partially purified from FDA 209-P (lanes 2 to 4 and 8, respectively) or 1, 2, 5, and 5 ng of *S. aureus* gyrase purified from *E. coli* (lanes 5 to 7 and 9, respectively) and either 700 mM KGlu (lanes 1 to 7) or no KGlu (lanes 8 and 9) were incubated, processed, and analyzed as described in Materials and Methods. I, I′, and II indicate the positions of form I (covalently closed supercoiled), form I′ (covalently closed relaxed), and form II (nicked circular) DNA, respectively.

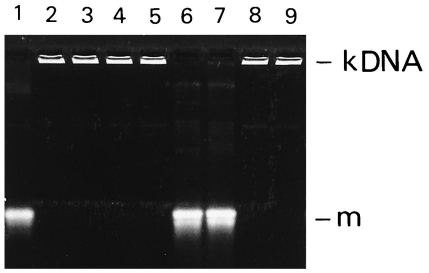

FIG. 3. Decatenation activity of *S. aureus* topoisomerase IV. Lanes 1 and 6, standard reaction mixture; lane 2, ATP omitted; lane 3, KGlu omitted; lane 4, GrlA omitted; lane 5, GrlB omitted; lane 7, with *E. coli* topoisomerase IV; lane 8, with GrlA-ParE mixture; lane 9, with ParC-GrlB mixture; m, monomeric minicircles.

Asp, D-Glu, and 2-methylglutamate could stimulate gyrase with almost the same efficiency as KGlu, but none of the following compounds was active: Ala, Arg, Gly, Lys, Pro, Ser, 4-aminobutyrate, glutarate, succinate, *trans*-glutaconate, γ-glutamylglutamate, and glycine betaine. Interestingly, the sodium and Tris salts of Glu had no stimulatory effect. Furthermore, at concentrations up to 800 mM KCl, no stimulatory effect was observed. The stimulation was therefore not restricted to glutamate but encompassed the general structure of dicarboxylic α-amino acids, regardless of the stereochemistry of the α-carbon. Gyrase extracted from *S. aureus* FDA 209-P showed exactly the same behavior (Fig. 2). On the other hand, *E. coli* gyrase was totally insensitive to stimulation by KGlu (data not shown).

The gyrase preparations produced in *E. coli* were apparently devoid of gyrase and/or topoisomerase IV from the host, as demonstrated in two different ways. First, gyrase preparations were unable to catalyze the fluoroquinolone-dependent cleavage of linear DNA under experimental conditions in which *E. coli* topoisomerases were fully active (see below). Second, a purification was carried out under standard conditions from 3 liters of *E. coli* BL21(DE3)(pET11a), a strain lacking *S. aureus gyrA* and *gyrB* but otherwise isogenic to *E. coli* BL21(DE3)(pXL2401). This process yielded a preparation showing no detectable protein bands with molecular weights the same as those of *E. coli* type II topoisomerase subunits and a nondetectable supercoiling activity (data not shown). From these data, the contamination of *S. aureus* gyrase by *E. coli* gyrase was estimated to be <0.1%.

Purification and characterization of *S. aureus* topoisomerase IV. GrlA and GrlB proteins were produced in a soluble form in *E. coli* XL1 Blue harboring the T7 promoter-based expression vectors pXL2340 and pXL2320 and were separately purified to more than 95% homogeneity (Fig. 1). The two GrlA and GrlB proteins had apparent M_rs of 90,000 and 75,000, respectively. Topoisomerase IV, which was reconstituted by combining stoichiometric amounts of the GrlA and GrlB subunits, showed a decatenation activity of 20,000 units/mg at the optimum KGlu concentration (350 mM) (Fig. 3) and a superhelical DNA relaxation activity of 30,000 U/mg in the absence of KGlu. These two activities were ATP dependent. No decatenation, relaxation, or supercoiling activity was observed with GrlA or GrlB alone. Decatenation activity was strongly dependent on the presence of KGlu (Fig. 3); the absence of KGlu resulted in a 50-fold reduction in decatenation activity. Conversely, DNA relaxation activity was inhibited threefold by 350 mM KGlu (data not shown).

When GrlA or GrlB was combined with ParE or ParC, respectively, no decatenation activity could be detected (Fig. 3), indicating a complete lack of interspecies in vitro complementation between topoisomerase IV subunits of *E. coli* and *S. aureus*. Under the incubation conditions used (150 ng of each subunit and 200 mM glutamate), both GrlAB and ParCE proteins fully decatenated 100% of the kDNA. This result is in agreement with in vivo data which indicate that the temperature-sensitive phenotype of *Salmonella typhimurium parC* and *parE* mutants is complemented by the *S. aureus grlA* and *grlB* genes only when the two genes are coexpressed (9).

A GrlA mutant protein with a Ser-80→Tyr substitution ($GrlA^{Y80}$) was purified from 3 liters of *E. coli* XL-1 Blue(pXL2742) (Fig. 1) and was combined with GrlB. The resulting topoisomerase IV had a relaxation activity of 30,000 U/mg and a decatenation activity of 20,000 U/mg, identical to those of the wild-type enzyme.

Sensitivities of catalytic activities of *S. aureus* gyrase and topoisomerase IV to fluoroquinolone and novobiocin inhibition. The inhibitory effects of four fluoroquinolones (ofloxacin, norfloxacin, sparfloxacin, and ciprofloxacin) on topoisomerase IV-dependent decatenation of kDNA and on gyrase-dependent DNA supercoiling are presented in Table 2. With IC_{50}s for decatenation being in the range of 4 to 12 μg/ml, *S. aureus* topoisomerase IV sensitivity to quinolones was very similar to that of *E. coli* topoisomerase IV. IC_{50}s for supercoiling inhibition were higher for *S. aureus* gyrase (from 12 μg/ml for sparfloxacin and ofloxacin to >100 μg/ml for norfloxacin), providing biochemical evidence that in *S. aureus*, topoisomerase IV is consistently more sensitive to fluoroquinolones than gyrase. By comparison, IC_{50}s for *E. coli* gyrase were ≤1.5 μg/ml. Experiments with the *S. aureus* mutant topoisomerase IV ($GrlA^{Y80}$-GrlB) clearly demonstrated that the S80Y substitution conferred to the enzyme an increased level of resistance to fluoroquinolones (Table 2). For instance, inhibition of decatenation activity required 80 times more sparfloxacin for the mutant enzyme (500 μg/ml) than for the wild-type enzyme (6 μg/ml). However, the strain harboring this mutant topoisomerase IV (strain 2-2) was only four times less sensitive to sparfloxacin than the wild-type strain, probably because gyrase becomes the actual target of sparfloxacin when topoisomerase IV is first protected from inhibition by mutation. From our results (Table 2), ciprofloxacin and norfloxacin showed a pattern of inhibition similar to that of sparfloxacin, indicating that these drugs primarily target topoisomerase IV in *S. aureus.*

For ofloxacin, on the other hand, the drug sensitivity of the mutant topoisomerase IV was reduced 25-fold in vitro, whereas the wild-type strain and strain 2-2 displayed the same sensitivity in vivo (2 μg/ml). Although the in vitro data suggest that *S. aureus* topoisomerase IV is a target of ofloxacin, the

TABLE 2. Inhibitory activities of quinolones and other topoisomerase-targeted drugs on gyrase, topoisomerase IV, and whole cells

Compound	IC_{50}, supercoiling (μg/ml)		IC_{50}, decatenation (μg/ml)			MIC (μg/ml)[a]	
	E. coli gyrase	*S. aureus* gyrase	*E. coli* topoisomerase IV	*S. aureus* topoisomerase IV	*S. aureus* topoisomerase IV ($GrlA^{Y80}$)	*S. aureus* FDA 574	*S. aureus* strain 2.2[b]
Ciprofloxacin	<0.75	25	2	4	60	0.5	8
Sparfloxacin	0.5	12	3.5	6	500	0.25	1
Norfloxacin	1.5	>100	7	12	125	1	32
Ofloxacin	<0.75	12	12	10	250	2	2
Novobiocin	ND[c]	ND	1.5	30	20	0.12	0.12

[a] From Ferrero et al. (7).
[b] $GrlA^{Y80}$ mutant.
[c] ND, not determined.

results obtained in vivo imply that ofloxacin does not primarily target topoisomerase IV. In this case, the primary target of ofloxacin may be gyrase.

With an IC_{50} of 30 μg/ml, *S. aureus* topoisomerase IV was 20-fold less sensitive to novobiocin than the *E. coli* enzyme. As expected, the mutant enzyme was not significantly affected in its sensitivity toward novobiocin.

Stimulation of gyrase- and topoisomerase IV-associated DNA cleavage by fluoroquinolones and other topoisomerase-targeted drugs. Experiments aimed at determining the levels of fluoroquinolone-induced DNA cleavage with topoisomerase IV essentially confirmed the results obtained by the catalytic assays, i.e., similar sensitivities of *E. coli* and *S. aureus* topoisomerase IV and a high level of resistance of the mutant with an Ser-80→Tyr mutation (Table 3). On the other hand, no stimulation of DNA cleavage by 25 μg of sparfloxacin or ciprofloxacin per ml could be detected with *S. aureus* gyrase, regardless of the concentration of KGlu used, whereas similar experiments with *E. coli* gyrase gave concentrations at which 50% of the linear plasmid substrate was cleaved of ≤0.05 μg/ml for these drugs (Fig. 4). In order to confirm this result, further cleavage reactions were carried out with supercoiled pBR322 plasmid DNA, in which the amount of the linear DNA produced represents the amount of topoisomerase-dependent cleavage products. Again, even in the presence of 100 μg of ciprofloxacin per ml, DNA cleavage could hardly be detected with *S. aureus* gyrase, whereas up to 35% of the initial supercoiled DNA was converted to a linear form with *S. aureus* topoisomerase IV, confirming that fluoroquinolones do not efficiently stabilize *S. aureus* gyrase-DNA covalent complexes in vitro.

The terpenoid antibiotic clerocidin (17) induced DNA cleavage with all the enzymes tested (Table 3) except with *S. aureus* gyrase, indicating that the absence of drug-induced DNA cleavage with this enzyme is not restricted to quinolones. Wild-type and $GrlA^{Y80}$ *S. aureus* topoisomerases IV were equally sensitive to clerocidin, and both the wild-type and the quinolone-resistant $GrlA^{Y80}$ *S. aureus* strains were equally susceptible to clerocidin (data not shown). Conversely, no mutation was found in the quinolone resistance-determining region of the topoisomerase IV A subunit of clerocidin-resistant *S. aureus* mutants (data not shown). Saintopin, an anticancer antibiotic belonging to the tetracenomycin family (35), stimulated cleavable complex formation with gyrase and topoisomerase IV from *E. coli* but was inactive against the corresponding *S. aureus* enzymes.

Binding of sparfloxacin to gyrase-DNA complexes. Ternary complexes involving *S. aureus* gyrase, DNA, and sparfloxacin were unambiguously evidenced by spin column chromatography (Fig. 5), although to a lesser extent than with *E. coli* gyrase. Assuming that all gyrase molecules were involved in ternary complexes, the stoichiometry of bound sparfloxacin:gyrase was 3.7 ± 0.6 in experiments with *E. coli* gyrase and 1.8 ± 0.3 in experiments with *S. aureus* gyrase. With both enzymes, complex formation was Mg^{2+} dependent. These results indicate that even though no detectable cleavage products could be observed in cleavage reactions with *S. aureus* gyrase (see above), a ternary complex which is stable enough to withstand

TABLE 3. Stabilization of covalent topoisomerase-DNA complexes by quinolones and other topoisomerase-targeted drugs

Compound	CC_{50} (μg/ml)[a]				
	E. coli gyrase	*S. aureus* gyrase	*E. coli* topoisomerase IV	*S. aureus* topoisomerase IV	*S. aureus* topoisomerase IV ($GrlA^{Y80}$)
Ciprofloxacin	<0.05	>25	0.1	0.1	1
Sparfloxacin	0.05	>25	0.5	0.5	25
Norfloxacin	ND[b]	ND	ND	0.25	10
Ofloxacin	ND	ND	ND	0.25	10
Clerocidin	0.5	>25	15	10–25	10–25
Saintopin	0.1	>10[c]	0.5	>10[c]	>10[c]

[a] CC_{50}, the drug concentration at which 50% of the linear plasmid substrate was cleaved.
[b] ND, not determined.
[c] Saintopin intercalation in DNA at concentration ≥10 μg/ml precluded determinations of the drug concentration at which 50% of the linear plasmid substrate was cleaved.

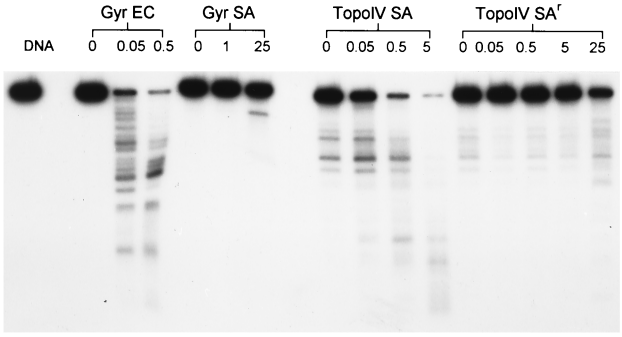

FIG. 4. Stabilization of topoisomerase-DNA covalent complexes by sparfloxacin. Reactions were carried out with linear ^{32}P-3′-end-labeled pBR322 as the substrate, the indicated concentration of sparfloxacin (in micrograms per milliliter), and either *E. coli* gyrase (Gyr EC) or *S. aureus* gyrase (Gyr SA) or wild-type *S. aureus* topoisomerase IV (TopIV SA) or *S. aureus* $GrlA^{Y80}$ topoisomerase IV (TopIV SA^r).

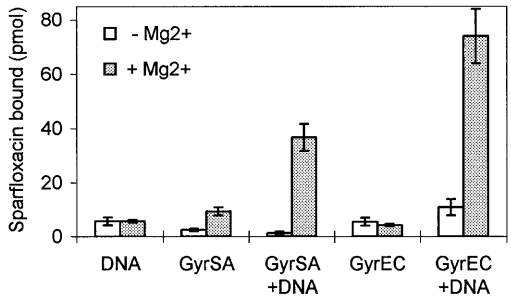

FIG. 5. Formation of enzyme-DNA-sparfloxacin complexes with *E. coli* or *S. aureus* gyrase. The amount of [4-^{14}C]sparfloxacin engaged in gyrase-pBR322 DNA-sparfloxacin ternary complexes was determined by liquid scintillation counting, after spin column chromatography. Control experiments in which gyrase, DNA, or Mg^{2+} was omitted were run in parallel. Results are the averages ± standard deviations of six experiments. GyrSA, *S. aureus* gyrase; GyrEC, *E. coli* gyrase.

isolation is formed between sparfloxacin, *S. aureus* gyrase, and DNA. In this complex, sparfloxacin might inhibit gyrase activity by interfering with the DNA breakage step.

DISCUSSION

Both gyrase and topoisomerase IV from *S. aureus* have been expressed in *E. coli*, purified to homogeneity, and characterized in a comparative manner. We obtained biochemical evidence showing that in *S. aureus*, topoisomerase IV is the primary target of fluoroquinolones such as ciprofloxacin, norfloxacin, and sparfloxacin, thus adding strong support to previous genetic studies that arrived at the same conclusion. In this bacterium, however, in view of the results obtained with ofloxacin, it appears that topoisomerase IV is not the primary target of this fluoroquinolone.

In *E. coli*, the situation is quite different. Quinolone agents primarily target gyrase (9, 29), and the inhibition of topoisomerase IV becomes apparent only when gyrase is mutated, thus making topoisomerase IV secondary to gyrase as a quinolone target (5, 14). Strikingly, *S. aureus* gyrase and topoisomerase IV display quite different behaviors toward fluoroquinolones compared with the behaviors of their *E. coli* homologs, i.e., higher sensitivity of topoisomerase IV to fluoroquinolones and the predominance of topoisomerase IV over gyrase as an in vivo target. This particular behavior of *S. aureus* is probably related to the failure to detect any stimulation of gyrase-associated cleavable complex with fluoroquinolones in vitro.

S. aureus gyrase and topoisomerase IV required high concentrations of KGlu (700 and 340 mM, respectively) for efficient catalytic activity in vitro, to the extent that DNA supercoiling and decatenation were reduced 500- and 50-fold, respectively, in the absence of KGlu. *S. aureus* is known to contain high intracellular concentrations of dicarboxylic amino acids, Glu and Asp, and the topoisomerases of this microorganism may well have evolved to accommodate this particular environment. However, the intracellular concentrations of Asp and Glu reported in the literature do not exceed 250 to 300 mM (3). This is, in any case, much lower than the concentrations required for the complete stimulation of gyrase. However, it cannot be excluded that Asp and Glu are unevenly distributed inside the cell, so that much higher concentrations would be reached locally, e.g., in regions surrounding DNA. The observation that Glu inhibits the relaxation activity but stimulates the decatenation activity of *S. aureus* topoisomerase IV indicates that this compound is not an activator of this enzyme but that it participates in some way in the decatenation reaction in a specific manner by favoring intermolecular DNA strand passage reactions through DNA breaks over their intramolecular counterparts. On the other hand, Glu stimulates gyrase-dependent supercoiling whose mechanism involves intramolecular DNA strand passage events. These apparent differences may be reconciled if we assume that the role of Glu (or Asp) in in vitro assays of *S. aureus* type II topoisomerases is to provide gyrase and topoisomerase IV with an environment which mimics the in vivo situation, in particular by favoring a spatial arrangement of plasmid DNA which stimulates interaction with topoisomerases and which promotes their physiological catalytic activities. The lack of a significant KGlu effect on topoisomerase IV-mediated cleavage provides evidence that KGlu does not simply elicit enhancement of topoisomerase binding on DNA but is more likely to stimulate a step further along the catalytic cycle, probably the strand passage reaction. Alternatively, the requirement for a high concentration of Asp-Glu in in vitro assays of *S. aureus* topoisomerases might be a substitute for a yet elusive protein that is associated with DNA and that interacts with topoisomerases in vivo. Anyhow, the marked stimulatory effect of glutamate on the catalytic activities of *S. aureus* gyrase and topoisomerase IV will have to be taken into consideration in further biochemical studies of *S. aureus* topoisomerases.

The availability of purified *S. aureus* type II topoisomerases opens the way to further exploration of the molecular mechanism of gyrase and topoisomerase IV inhibition by quinolone drugs and to obtaining a better understanding of the large differences displayed by the *S. aureus* topoisomerases compared with their *E. coli* counterparts. In particular, further studies can now be carried out in order to identify the elusive stimulatory compound that acts on *S. aureus* topoisomerases. Ultimately, these studies could explain why no stimulation of the cleavable complex is observed with gyrase in the presence of fluoroquinolones.

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