Purification and Characterization of Recombinant *Staphylococcus haemolyticus* DNA Gyrase and Topoisomerase IV Expressed in *Escherichia coli*

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The subunits of DNA gyrase and topoisomerase IV from *Staphylococcus haemolyticus* were expressed in *Escherichia coli*, purified to homogeneity, and used to reconstitute active enzymes that were sensitive to known topoisomerase inhibitors. This represents the first description of a method for isolating type II topoisomerases of a coagulase-negative staphylococcal species.

Coagulase-negative staphylococci (CoNS) are ubiquitous human microbes that are the most commonly isolated bacteria in clinical microbiology laboratories. Once thought of as avirulent and often dismissed as culture contaminants, these commensal organisms are becoming more frequently recognized as opportunistic pathogenic agents. In particular, CoNS have been associated with nosocomial infections among immunocompromised individuals, high-risk neonates, and hospitalized patients with imbedded foreign bodies (2, 8, 10, 16, 17, 19, 25). Moreover, treatment of CoNS infections has become increasingly difficult due to the growing prevalence of multiple-antibiotic-resistant phenotypes in clinical isolates (1, 12). One well-characterized species within this group is *Staphylococcus haemolyticus*, a normal inhabitant of the human skin. While generally considered to be nonpathogenic, there is growing evidence that *S. haemolyticus* is a causative agent of human disease (4, 9, 20), and as with other CoNS, clinical isolates of *S. haemolyticus* increasingly display a multiple-antibiotic-resistant phenotype (1, 6, 20, 21, 24).

The escalation of antibiotic resistance observed in clinical isolates of CoNS can be attributed to the widespread and frequently indiscriminant use of antibiotics to treat coagulase-positive *Staphylococcus aureus* infections in nosocomial patient populations. Since fluoroquinolone antibiotics are commonly used to treat such infections, clinical resistance to these agents in particular has increased dramatically in CoNS in recent years (5, 13, 21). The therapeutic targets of fluoroquinolones are DNA gyrase and topoisomerase IV, two type II topoisomerases that mediate distinct functions within bacterial cells (11). DNA gyrase is responsible for maintaining the topological state of DNA during replication and is the only enzyme known to introduce negative supercoils into DNA. In contrast, topoisomerase IV is a cellular decatenase that separates daughter chromosomes following a round of replication. Both DNA gyrase and topoisomerase IV are heterodimeric enzymes

composed of two subunits that form an A₂B₂ complex and require the free energy of ATP hydrolysis to drive their respective catalytic activities. While these proteins are highly conserved among all bacterial species and have been studied extensively at the biochemical level, the purification and characterization of a DNA gyrase or topoisomerase IV from a CoNS species had not been described to date. Consequently, in this study recombinant subunits of *S. haemolyticus* topoisomerases were expressed and purified in an attempt to reconstitute active enzymes for use in *in vitro* inhibition assays and other biochemical work.

Expression of the A and B subunits of *S. haemolyticus* DNA gyrase and topoisomerase IV as recombinant proteins in *Escherichia coli*. The *gyrA* and *gyrB* genes encoding the A and B subunits of DNA gyrase, respectively, and the *griA* and *griB* genes encoding the A and B subunits of topoisomerase IV, respectively, were identified within *S. haemolyticus* genomic sequences of the PathoSeq database (version 4.1, September 2001; Elitra Pharmaceuticals, Inc., San Diego, Calif.). The PCR was used to amplify each of these open reading frames from *S. haemolyticus* genomic DNA, using the following primer pairs: 5'-ATGGCTGACTTACCTCAATCAAG-3' (forward) and 5'-GGGAAGTCTTGTGTTTGAAGG-3' (reverse) for *gyrA*, 5'-ATGGTGAATACATTGTCAGATGTAAAC-3' (forward) and 5'-CTACTATTAGAAATCCAAGTTCGCATATA C-3' (reverse) for *gyrB*, 5'-ATGAGTGAGATAATTCAAGATTATC-3' (forward) and 5'-CTACTATTAGATTGTCATATCTAAGACGTC-3' (reverse) for *griA*, and 5'-ATGCATTACTCAGATGATTCTATTC-3' (forward) and 5'-TTATTCCTCCTCA GTATATTCTTC-3' (reverse) for *griB*. Genomic DNA was isolated from an *S. haemolyticus* ATCC 29970 culture grown in Trypticase soy broth at 37°C, using the DNeasy tissue kit according to the manufacturer's instructions (QIAGEN, Valencia, Calif.), except that lysozyme was replaced by lysostaphin (Sigma Chemical Company, St. Louis, Mo.) at a final concentration of 0.1 mg/ml for a 30-min incubation at 37°C. All PCRs were carried out with 100 ng of purified genomic DNA, 25 μM forward and reverse primers, 200 μM deoxynucleoside triphosphate mix, 1 mM MgSO₄, and PLATINUM Pfx DNA polymerase and buffer following the manufacturer's instructions (Invitrogen Corporation,

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Carlsbad, Calif.), except that the *gyrA* reaction required the addition of *Pfx* Enhance solution (Invitrogen Corporation) at a concentration of 1×. PCR products were then A-tailed by the addition of recombinant Taq DNA polymerase to the reaction mixture followed by incubation at 72°C for 10 min. Each PCR product was then cloned directly into a pCRT7/CT-TOPO expression vector (Invitrogen Corporation) by immediately mixing 1 µl of PCR product, 1 µl of expression vector, 1 µl of TOPO salt solution, and 3 µl of water. Following a 5-min incubation at room temperature, the resulting product was transformed into chemically competent One Shot *E. coli* TOP10F' cells (Invitrogen Corporation). Plasmids containing inserts of the correct size and orientation as determined by restriction site mapping were then confirmed by DNA sequencing (MWG Biotech, Inc., High Point, N.C.). The cloned topoisomerase subunit genes were found to be identical to the corresponding open reading frames present in the PathoSeq database, with the following exceptions: (i) the TTG and AAT start codons of *grlA* and *grlB*, respectively, were changed to ATG by using an altered sequence in the forward PCR primers with the intention of maximizing gene expression in the *E. coli* expression host; (ii) the *gyrA* construct contained an additional 68 bp of sequence 3' to the stop codon, which was required for matching the G+C contents of the forward and reverse primers used in the PCR; and (c) the *grlB* construct was missing the final two amino acids of the PathoSeq open reading frame, which may not be present in all *S. haemolyticus* isolates as they map to a 17-residue segment that is not conserved in the *grlB* genes of other gram-positive species.

Expression constructs containing native, full-length, untagged sequences of the *gyrA*, *gyrB*, and *grlA* genes were transformed into BL21-Gold (DE3) pLysS-competent cells (Stratagene, La Jolla, Calif.), whereas the expression construct containing the native, full-length, untagged sequence of the *grlB* gene was transformed into *E. coli* BL21-Gold (DE3)-competent cells (Stratagene). Transformants were grown overnight at 37°C on Luria-Bertani (LB) agar plates containing 0.15-mg/ml ampicillin. The resulting colonies, which were used to inoculate LB broth supplemented with 0.2-mg/ml carbenicillin and 50-µg/ml chloramphenicol (omitted for *grlB* transformants), were then grown to log phase at 30°C and stored overnight at 4°C. This culture was centrifuged at 10,000 × *g* for 5 min, and the cell pellet was resuspended in fresh LB medium supplemented with 0.2-mg/ml carbenicillin. A portion of this cell suspension was then transferred to a larger culture of LB medium supplemented with 0.2-mg/ml carbenicillin and grown to log phase at 30°C. Expression of the protein subunits was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture followed by an additional 2 h of incubation at 30°C. The induced cells were then recovered by centrifugation, washed with an ice-cold solution of 50 mM Tris-HCl (pH 8.0) and 2 mM EDTA, and stored as a pellet at −80°C.

Purification of recombinant A and B subunits of *S. haemolyticus* DNA gyrase and topoisomerase IV. Frozen cell pellets were resuspended in TED buffer (50 mM Tris-HCl [pH 7.6], 1 mM EDTA, 5 mM dithiothreitol [DTT]) containing 0.5 mM phenylmethylsulfonyl fluoride and 1-mg/ml lysozyme. Following a 30-min incubation on ice, the cells were lysed by Dounce homogenization. The resulting lysate was probe sonicated to reduce viscosity, centrifuged to remove insoluble material, di-

FIG. 1. SDS-PAGE analysis of recombinant A and B subunits of *S. haemolyticus* DNA gyrase and topoisomerase IV. A total of 0.5 µg of the final protein preparations of each topoisomerase subunit was electrophoresed on 8% SDS-PAGE gels and stained with SimplyBlue SafeStain (Invitrogen Corporation). The mobility of molecular mass markers (in kilodaltons) is shown to the left.

alyzed into TGED buffer (TED buffer containing 10% glycerol), and then subjected to streptomycin sulfate precipitation (1% final concentration). Precipitated protein was recovered by centrifugation and dissolved in TGED buffer containing 1 M NaCl. Insoluble material was again removed by centrifugation, and the clarified supernatant was subjected to ammonium sulfate precipitation (60% saturation). This precipitated protein was recovered by centrifugation, dissolved in TGED buffer, and dialyzed overnight into TGED buffer. The clarified lysate was applied to a HiPrep 16/10 heparin FF column (Amersham Biosciences Corporation, Piscataway, N.J.) that was preequilibrated in TGED buffer. Bound protein was eluted with a gradient of 0 to 0.6 M NaCl in TGED buffer. Fractions that contained the subunit of interest as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were pooled, dialyzed, and applied to a Mono Q HR 10/10 column (Amersham Biosciences Corporation, Piscataway, N.J.) that was preequilibrated in TGED buffer. The bound subunit resolved into a single protein band following elution with a gradient of 0 to 0.45 M NaCl in TGED buffer. Fractions containing the purified protein were pooled; diluted with 1 volume of 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 200 mM KCl, and 20% glycerol (to give concentrations of 50 mM Tris-HCl [pH 7.6], 1 mM EDTA, 2.5 mM DTT, 100 mM KCl, and 10% glycerol in the final protein preparation); and then stored at −80°C. Unless noted otherwise, all steps of the purification process were performed at 4°C.

The purity and molecular mass of each subunit in the final protein preparations were evaluated by SDS-PAGE. Each of the four subunits was determined to be greater than 95% pure, and their apparent molecular masses were consistent with the predicted molecular masses of the polypeptides encoded by *gyrA* (101 kDa), *gyrB* (73 kDa), *grlA* (90 kDa), and *grlB* (74 kDa) (Fig. 1). The yields ranged from 6 to 19 mg of purified protein per liter of culture, and none of the purified subunits

FIG. 2. Agarose gel electrophoresis of DNA gyrase and topoisomerase IV assays containing recombinant *S. haemolyticus* enzymes. (A) DNA gyrase assays. All reactions contained relaxed closed circular pBR322 plasmid as the substrate, and the reaction mixture in lane 3 contained 0.7 μ M coumermycin A1 as an inhibitor. The mobility of the relaxed DNA substrate (R) and the supercoiled DNA product (S) are indicated at the right. (B) Topoisomerase IV assays. All reactions contained interlinked kinetoplast DNA minicircles as the substrate, and the reaction mixture in lane 3 contained 6.2 μ M coumermycin A1 as an inhibitor. The mobilities of the linked DNA substrate (L) and the free DNA product (F) are indicated to the right.

exhibited detectable DNA-independent ATPase activity (J. C. Bronstein and S. L. Olson, unpublished data).

Characterization of the enzymatic properties of recombinant *S. haemolyticus* DNA gyrase and topoisomerase IV. Enzymatic activities of topoisomerases reconstituted from their purified subunits were monitored by either the introduction of supercoils into relaxed closed circular pBR322 plasmid DNA (the DNA gyrase assay) or the decatenation of interlinked kinetoplast DNA minicircles (the topoisomerase IV assay). The DNA gyrase assay contained 35 mM Tris-HCl (pH 7.5), 250 mM potassium glutamate, 5 mM $MgCl_2$, 2 mM DTT, 50- μ g/ml bovine serum albumin, 1 mM ATP, 250 ng of relaxed pBR322 DNA (TopoGEN, Inc., Columbus, Ohio), and 50 ng of each gyrase subunit in a 30- μ l reaction mixture that was incubated at 37°C for 45 min. The reaction was terminated by the addition of 7 μ l of a stop solution containing 1.5 μ l of 10% SDS, 1.5 μ l of 10-mg/ml proteinase K, and 4 μ l of 10 $\times$ Blue-Juice gel loading buffer (Invitrogen Corporation). The topoisomerase IV assay was identical to the DNA gyrase assay, except that it employed 120 ng of kinetoplast DNA (TopoGEN, Inc.) as a substrate, 20 ng of each topoisomerase IV subunit, and a 15-min incubation time. The products of either

assay were resolved on 0.8% agarose gels, stained with ethidium bromide, and quantitated on a Gel Dock 2000 gel documentation system (Bio-Rad Laboratories, Hercules, Calif.). In experiments that included topoisomerase inhibitors, the percent supercoiled pBR322 or percent decatenated kinetoplast DNA minicircles relative to drug-free control reactions was calculated for each inhibitor concentration; these were then plotted against the log of the respective inhibitor concentrations, and 50% inhibitory concentrations (IC_{50} s) were calculated from the resulting curves.

Both of the recombinant *S. haemolyticus* topoisomerases were found to be enzymatically active, as evidenced by the ability of reconstituted DNA gyrase and topoisomerase IV to supercoil relaxed pBR322 DNA and decatenate kinetoplast DNA, respectively (Fig. 2). The specific activities for DNA gyrase and topoisomerase IV were calculated to be 22 and 180 U/ μ g, respectively, where a unit of topoisomerase activity is defined as the amount of enzyme needed to supercoil or decatenate 50% of substrate in 1 h at 37°C under the assay conditions described above (3, 18). These topoisomerases were also shown to exhibit sensitivity to a panel of fluoroquinolone and coumarin antibiotics (Table 1 and Fig. 2), further confirming the authenticity of their observed enzymatic properties. The IC_{50} s of these inhibitors against *S. haemolyticus* DNA gyrase and topoisomerase IV were found to be comparable to those reported for the corresponding enzymes from *S. aureus* (3, 7, 18, 22, 23). Additionally, in the presence of ciprofloxacin and clinafloxacin, a linear DNA product indicative of cleavage complex formation could be detected within reaction mixtures following agarose gel electrophoresis (J. C. Bronstein and M. Tomilo, unpublished data). Whole-cell inhibitory activities against *S. haemolyticus* (Table 1), as determined by susceptibility testing following National Committee for Clinical Laboratory Standards guidelines (14, 15), were also found to be comparable to those reported from *S. aureus* (3, 18, 23). These results suggest that *S. haemolyticus* topoisomerases may someday prove to be a useful surrogate for their *S. aureus* counterparts in routine biochemical assays.

In summary, this report describes a simple and reliable procedure for the expression and purification of the subunits that comprise the DNA gyrase and topoisomerase IV of *S. haemolyticus* and their reconstitution into fully active enzymes. This represents the first characterization of topoisomerases from a species of CoNS, which are now recognized as a growing source of opportunistic infection in hospital settings. The ability to purify topoisomerases from *S. haemolyticus* and other CoNS, including fluoroquinolone-resistant strains, should provide researchers with new biochemical tools with which to assess the efficacy of current and future antibiotics targeting this class of enzymes.

Nucleotide sequence accession number. Sequences for the *S. haemolyticus* *gyrA*, *gyrB*, *grlA*, and *grlB* genes are available in GenBank under accession no. AY341071, AY341072, AY341073, and AY341074, respectively.

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TABLE 1. Whole-cell and in vitro activities of fluoroquinolone and coumarin antibiotics against *S. haemolyticus* and its topoisomerases

Compound	IC_{50} (μ M) ^a		MIC ₉₀ (μ g/ml) for <i>S.</i> <i>haemolyticus</i> ^b
	DNA gyrase	Topoisomerase IV	
Ciprofloxacin	20.0 (14.0)	8.39 (6.74)	0.5
Clinafloxacin	1.97 (0.62)	2.47 (1.74)	0.06
Novobiocin	0.08 (0.02)	98.5 (64.2)	0.5
Coumermycin A1	0.20 (0.07)	0.91 (0.49)	0.015

^a Standard deviations (included in parentheses) were calculated by using the results of five assays per experiment.

^b MIC₉₀, MIC at which 90% of the isolates tested are inhibited. These values were generated with strain ATCC 29970 SH-1812.

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