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포도상구균으로부터  
DNA topoisomerase I 유전자의  
클로닝 및 특성에 관한 연구

Molecular cloning and characterization of  
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*Staphylococcus aureus*

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## ABSTRACT

### Molecular cloning and characterization of a type I DNA topoisomerase gene from *Staphylococcus aureus*

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Type I topoisomerase plays critical roles in DNA metabolism and cell survival. In this study, type I topoisomerase gene from *Staphylococcus aureus* sp. strain C-66 cells was cloned in pBAD /His A expression vector and expressed in *E. coli* Top 10 cells. The coding region of this gene was 2,070 nucleotides capable of encoding a polypeptide of 690 amino acids with a predicted molecular mass of 79.1 kDa. The recombinant plasmid named pTP I expressed active type I topoisomerase upon induction with 0.02% L-arabinose. The topoisomerase expressed from pTP I plasmid in *E. coli* was purified through an affinity chromatography on Hitrap chelating column. The topoisomerase activity of the

purified enzyme was  $Mg^{2+}$ -dependent and ATP-independent when supercoiled DNA was used as a substrate. The enzyme could relax only negatively supercoiled DNA, not positively supercoiled DNA. The optimal temperature and pH for the enzyme activity were 37°C and 7.5, respectively. The activity of enzyme was significantly activated in the presence of 50 mM NaCl. The enzyme activity could be clearly inhibited by treatment with camptothecin, but not by nalidixic acid, etoposide, and spermidine. The enzyme made a single-stranded nick on negatively supercoiled DNA and the 5' end of the nick could covalently linked with the enzyme. All these results suggest that the purified enzyme is a typical type I DNA topoisomerase.

## I. INTRODUCTION

DNA topoisomerase is a broad group of enzymes with the ability to manipulate the topological state of DNA. They catalyze the interconversion of topological isomers of DNA molecules and have been identified and purified from both prokaryotic and eukaryotic organisms. These enzymes modulate the topology of DNA during process such as replication, transcription and recombination. These enzymes also introduce a transient break in the phosphodiester backbone through formation of a covalent protein DNA intermediate and allow the DNA strands to pass through one another. These enzymes alter the linking number of DNA by catalyzing a three-step process. The first, the cleavage of one or both strands of DNA. The second step, the passage of a segment of DNA through this break and the last step is the resealing of the DNA break.

Based on their catalytic mechanisms, topoisomerases are classified into two classes, type I and type II, which are distinguished by their ability to cleave one or both strands of a DNA duplex (Wang, 1996 and 2002; Champoux, 2001). Type I DNA topoisomerases cleave a single DNA strand and allow "controlled rotation" of the strand to relieve torsional stress one linking number at a time (Champoux, 1994; Gupta , 1995; Stewart , 1998), which effect topological changes in DNA by transiently cleaving one DNA strand at a time to allow the passage of

another strand (Maxwell and Gellert 1986). These result in the change in linking number of DNA by steps of one. Whereas type II DNA topoisomerases cleave both DNA strands and change the linking number by two by passing intact, double stranded DNA through the cut (Chen, 1994; Corbett, 1993).

Type I topoisomerases are further divided into two structurally and mechanistically distinct subfamilies on the basis of the polarity of enzyme attachment to the broken strands (i.e., IA, IB) that share no structural similarity and differ in reaction chemistry. The relaxation activity of type I DNA topoisomerase is controlled by two methods. one is the enzyme-bridged mechanism, the other is the strand-rotation mechanism (Dekker, 2002). The enzyme-bridged mechanism for type IA topoisomerases have been proposed to function by binding to a locally denatured region of a double-stranded DNA helix, transiently nicking one of the stands, passing the uncut strand through the nick, and resealing the broken strand. In the DNA cleavage stage, the protein DNA covalent intermediate is formed between a tyrosyl residue and the 5'-phosphate at the DNA break site. These enzymes apparently work as monomers and are ATP independent, so the directionality of each reaction is determined by the free energies of the segments of DNA involved. Whereas the strand rotation mechanism for type IB topoisomerase results in which the enzyme breaks one strand of the DNA helix by addition across a phosphodiester bond, allowing limited rotation about the intact

strand to relax several supercoils before resealing occurs. In contrast to the previous group, they bind preferentially to double-stranded DNA, and cleave one of the DNA strands of the duplex by forming a covalent protein DNA intermediate between a tyrosyl residue and the 3'-phosphate at the break site. During the DNA cleavage stage, the unbroken strand can pass through this enzyme-operated nick and release the twisting stress of a DNA double helix (Champoux, 1990). Type IB topoisomerases can completely relax both overwound and underwound DNA duplexes, plus indications that in the DNA cleavage stage these enzymes do not hold the 5'-end of the broken DNA strand (McCoubrey and Champoux, 1986).

Type II topoisomerases catalyze the ATP-dependent transport of one DNA duplex through a second DNA segment via a transient double-strand break (Wang, 1998). They carry out strand passage by first generating a transient double-strand (ds) DNA break in a 'gate' or G-segment through nucleophilic attack on the DNA backbone and the formation of 5'-phosphotyrosyl enzyme-DNA linkages. The broken G-segment ends are then separated, a second duplex (the 'transfer' or T-segment) passed through the break and the broken G-segment duplex resealed.

*Staphylococcus aureus* is a gram-positive bacterium that causes a variety of different human diseases and one of the major causes of community-acquired and hospital-acquired infections. It produces numerous toxins including super antigens that cause

unique disease entities such as toxic-shock syndrome and Staphylococcal scarlet fever, and has acquired resistance to practically all antibiotics. Its main habitats are the nasal membranes and skin of warm-blooded animals, in whom it causes a range of infections from mild, such as skin infectious and food poisoning, to life-threatening, such as pneumonia, sepsis, osteomyelitis, and infectious endocarditis. The organism produces many toxins and is highly efficient at overcoming antibiotic effectiveness.

This study was performed to clone and characterize a gene encoding topoisomerase from *Staphylococcus aureus* sp. strain C-66. The topoisomerase gene was amplified by polymerase chain reaction (PCR) using a pair of specific PCR primers from the Staphylococcal strain, cloned into pBAD/His A vector, and expressed in *E. coli* cells. The expressed protein was purified using an affinity chromatography on Hitrap chelating column. In addition biochemical properties of the purified enzyme were also characterized and the physiological function of the purified enzyme related to the change of DNA topology was investigated.

**Table 1 . The three different type of DNA topoisomerase families**

Family	Characteristics	Enzyme	Gene	Organism
Type IA	<ul style="list-style-type: none"> <li>• Cleave single DNA strands</li> <li>• Forming a 5'-phosphotyrosine covalent intermediate</li> </ul>	<ul style="list-style-type: none"> <li>• Bacterial DNA topoisomerase I</li> <li>• Bacterial DNA topoisomerase III</li> <li>• Eukaryotic DNA topoisomerase III</li> <li>• Reverse gyrase</li> </ul>	<p><i>TOPA</i></p> <p><i>TOPB</i></p> <p><i>TOP3</i></p>	<p><i>E. coli</i></p> <p><i>E. coli</i></p> <p><i>S. cerevisiae</i></p> <p><i>S. acidocaldarius</i></p>
Type IB	<ul style="list-style-type: none"> <li>• Bind duplex DNA cleave one of the strands, forming a 3'-phosphotyrosine covalent intermediate</li> </ul>	<ul style="list-style-type: none"> <li>• Eukaryotic DNA topoisomerase I</li> <li>• DNA topoisomerase V</li> <li>• Vaccinia virus topoisomerase</li> <li>• Variola virus topoisomerase</li> <li>• Shope fibroma virus topoisomerase</li> </ul>	<p><i>TOP1</i></p>	<p><i>S. cerevisiae</i></p> <p><i>M. kandleri</i></p>
Type II	<ul style="list-style-type: none"> <li>• Cleave both strands of duplex DNA</li> <li>• Forming a pair of 5'-phosphotyrosine covalent intermediates</li> <li>• Function as dyadic enzymes and ATP dependent</li> </ul>	<ul style="list-style-type: none"> <li>• Bacterial DNA gyrase</li> <li>• Bacterial DNA topoisomerase IV</li> <li>• Eukaryotic DNA topoisomerase II</li> <li>• Bacteriophage T4 topoisomerase</li> <li>• African swine fever virus topoisomerase</li> </ul>	<p><i>GyrA+GyrB<math>\alpha</math></i></p> <p><i>ParC+ParE<math>\beta</math></i></p> <p><i>TOP2</i></p> <p><i>TOP2</i></p> <p><i>TOP2</i></p> <p><i>TOP2</i></p> <p><i>gn39+gn60+gn52</i></p>	<p><i>E. coli</i></p> <p><i>E. coli</i></p> <p><i>S. cerevisiae</i></p> <p><i>D. melanogaster</i></p> <p><i>H. sapiens</i></p> <p><i>H. sapiens</i></p>

*E. coli*, *Escherichia coli*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. acidocaldarius*, *Sulphobus acidocaldarius*; *D. melanogaster*, *Drosophila melanogaster*; *H. sapiens*, *Homo sapiens*; *M. kandleri*, *Methanopyrus kandleri*



## II. Materials and Methods

### II-1. Bacterial strains, plasmids and materials

*S. aureus* sp. strain C-66 cells were kindly given by Prof. Lim (Chosun University, Korea). *E. coli* strain DH 5 $\alpha$  was purchased from Hanahan and the pBAD/His A vector was purchased from Invitrogen (USA). Reagents for SDS-PAGE, protein molecular weight standards, etoposide, camptothecin, EDTA, nalidixic acid, spermidine, ethidium bromide (EtBr), DTT, PMSF, BSA, SDS, TEMED, N, N-methylene-bis-acrylamide, acrylamide, agarose and Trizma base were purchased from Sigma Co. (St. Louis, USA). PD-10 desalting column and Hitrap chelating column were purchased from Amersham Pharmacia Biotech Co. (Uppasla, Sweden). Bradford protein assay kit, and molecular size marker were obtained from Bio-Rad. (California USA). Klenow fragment and T4 polynucleotide kinase were purchased from New England Biolabs (USA). The bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* strain Top 10 used as host cells for amplification and transformation of recombinant plasmid.

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmids	Relevant genotype or description	Source or reference
<b>Strain</b>		
<i>Staphylococcus aureus</i> sp. strain C-66	Wild type	Prof. Y. Lim (Chosun university of Medicine collage)
<b><i>E. coli</i> strain</b>		
Top 10	F' mrcA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(araA-leu)7697 galU galK rpsL(Str <sup>R</sup> ) endA1 nupG	Invitrogen
DH5α	SupE44 ΔlacU169 (8LacZ ΔM15 ) hsdR17 recA1 gyrA96 thi-1 relA1	Hanahan
<b>plasmid</b>		
pBAD/His A	P <sub>ara</sub> BAD, araC, histidine fusion vector, Amp <sup>R</sup>	Invitrogen
pGEM3zf(-)	Transcription vector earring the T7 and sp6 promoters, Amp <sup>R</sup>	Invitrogen

## II-2. Cultivation of *Staphylococcus aureus* and *E. coli* cells.

*E. coli* strain Top 10 cells were grown on LB (Luria and Bertani, 1 g Bacto-tryptone, 0.5 g Bacto-yeast extract, 1 g NaCl) and *Staphylococcus aureus* C-66 cells were grown on 3% Tryptic soy broth. The cells were cultured at 37°C with vigorous shaking.

## II-3. Molecular cloning of a gene encoding topoisomerase I from *Staphylococcus aureus* sp. strain C-66.

A DNA fragment encoding *Staphylococcus aureus* topoisomerase I was amplified by polymerase chain reaction using genomic DNA template, Vent DNA polymerase (New England Biorab) with the following pair of primers : 5'-TGCACTCGAGATGGCAGATAATTTAGT CATTG-3' as forward primer containing a *Xho* I restriction site and 5'-CGGGGTACCTTATTTCTGCGCTGCCTCTTTAT-3' as reverse primer containing a *Kpn* I restriction site (*Xho* I and *Kpn* I sites are underlined). PCR was carried out on Applied Biosystem 9700 for 1 min at 94°C, 40 s at 55°C and 2 min 30 s at 72°C for 30 cycles. The PCR products were purified with PCR purification kit (QIAGEN). The PCR products were inserted into the pBAD/His A expression vector at the *Xho* I/*Kpn* I site and constructs were checked by DNA sequencing.

#### II-4. Protein expression of recombinant clone

A single colony of recombinant clone was inoculated into 5 ml of LB medium (1% LB broth, 1% yeast extract and 0.5% NaCl) containing ampicillin (50 µg/ml) at 37°C for overnight. The inoculated cells were put into 6 l of LB media containing 50 µg/ml ampicillin until  $A_{600}$  reached 0.5. Induction was carried out with 0.02% L-arabinose for 2 hr at 37°C. About 15 g of cells were obtained from 6 l of culture.

#### II-5. Purification of the recombinant topoisomerase I

All purification procedures were performed at 4°C. A single colony of recombinant clone was inoculated up to 0.5 at  $A_{600}$ . Add 0.02% of L-arabinose in the culture to induce the protein expression for 2 hr. The cells were harvested from 6 l of culture by centrifugation at 4500 g for 30 min and then the cells resuspended in 100 ml of lysis buffer (25 mM Tris-HCl, pH 7.5, 10 % (v/v) glycerol and 0.5 mM PMSF) and passed five times through a french pressure cell at 12,000 psi. The cell lysate was centrifuged at 10,000 g for 30 min at 4 °C and the supernatant was collected as a cell-free extract. Ammonium sulfate was added to the cell-free extract to give 30% saturation and the protein precipitate was removed by centrifugation at 10,000 g for 20 min. The supernatant was collected and the ammonium sulfate

concentration was increased to 70%. The precipitate was collected by centrifugation at 10,000 g for 20 min and dissolved in 20  $\mu\text{l}$  of buffer A (25 mM Tris-HCl, pH 7.5, 10%(v/v) glycerol, 0.5 mM PMSF and 20 mM imidazol). After centrifugation at 10,000 g for 30 min, the supernatant was desalted on a PD-10 column and then loaded onto a Hitrap chelating column equilibrated with buffer A. The column was washed with 10 column volumes of buffer A and the bound proteins were eluted with buffer B (25 mM Tris-HCl, pH 7.5, 10%(v/v) glycerol, 0.5 mM PMSF and 500 mM imidazol) under a linear gradient of imidazol from 20 to 500 mM. The chromatography was carried out with the FPLC system (Amersham Biosciences). Fractions with topoisomerase activity were pooled, concentrated and used as the purified enzyme.

#### II-6. Assay of the relaxation activity on the purified activity

Topoisomerase I activity was measured by the relaxation of supercoiled plasmid pGEM3zf(-). The assay mixture contained 200 ng of DNA, 50 mM Tris-HCl (pH 8), 0.5 mM EDTA, 30  $\mu\text{g}/\text{ml}$  BSA, 0.5 mM DTT and 1  $\mu\text{g}$  of enzyme. The sample incubated for 30 min at 37°C and the reaction stopped by the addition of 5  $\mu\text{l}$  of stop solution (5% SDS and 50 mM EDTA). The reaction products were electrophoresed onto 1.2% agarose gel without EtBr. After the electrophoresis, the gel stained with 0.5  $\mu\text{g}/\text{ml}$  of EtBr to visualize the DNA bands. Quantitative

analysis was performed by densitometric scanning of the negatives as described previously. One unit of topoisomerase activity was defined as the amount of enzyme required to relax 50% of the supercoiled pGEM3zf(-) plasmid DNA in the standard assay.

#### II-7. SDS-PAGE (SDS-polyacrylamide gel electrophoresis)

SDS-PAGE polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Samples were completely denatured in SDS sample buffer (1.25% Tris-HCl, pH 6.8, 20% glycerol, 2%  $\beta$ -mercaptoethanol, 0.1% bromophenol blue, 10% SDS) by boiling in a water bath for 2 min and electrophoresed on 10% to 12% acrylamide gel. The gels were stained with coomassie blue solution (0.005% coomassie blue R-250 in solution A) for 1 hr. The gels were then destained with destain solution (50% Methanol, 10% acetic acid).

#### II-8. Determination of protein concentration

The concentration of protein was determined by the method of Bradford (1976) using BSA as the standard. The 250  $\mu$ l of various volume of protein solution was put into 1.5 ml of microcentrifuge tube and then 250  $\mu$ l of Bradford reagent was added to reaction

mixture and vortexed. Two hundred  $\mu\text{l}$  of the mixture sample was put onto 96 well plate. The amount of the protein was measured by reading absorbance at 595 nm after 2 min.

#### II-9. Measurement of topoisomerase activity depending on time

The standard assay mixture contained with 200 ng of negatively supercoiled pGEM3zf(-) DNA, 2  $\mu\text{l}$  of 10x reaction buffer (50 mM Tris-HCl, pH 8.0, BSA (1 mg/ml), 1 mM DTT, 2.5 mM EDTA, pH 8.0, and 5 mM  $\text{MgCl}_2$ ) and 0.5  $\mu\text{l}$  of the sample to be tested and adjusted to a final volume of 20  $\mu\text{l}$ . The mixture was incubated for 5, 10, 15, 20, 25, 30 and 60 min at 37°C . The reaction was terminated by addition of 5  $\mu\text{l}$  of stop reagent (0.5 M EDTA, pH 8.0). Loading buffer was added and sample was run on 1.3% agarose gel without EtBr. After electrophoresis, the gel was stained with EtBr (0.5  $\mu\text{g/ml}$ ) and photographed under UV-illumination.

#### II-10. Measurement of topoisomerase activity depending on pH

The standard assay mixture contained with 200 ng of negatively supercoiled pGEM3zf(-) DNA, 2  $\mu\text{l}$  of 10x reaction buffer (50 mM Tris-HCl, pH 8.0, BSA (1 mg/ml), 1 mM DTT, 2.5 mM EDTA, pH 8.0 and 5 mM  $\text{MgCl}_2$ ) and 0.5  $\mu\text{l}$  of the sample to be tested and

adjusted to a final volume of 20  $\mu\text{l}$ . The mixture was incubated for 30 min at 37°C on different pHs conditions (3.0, 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0 and 11.0). The reaction was terminated by addition of 5  $\mu\text{l}$  of stop reagent (0.5 M EDTA, pH 8.0). Loading buffer was added and sample was run on 1.3% agarose gel without EtBr (0.5  $\mu\text{g}/\text{ml}$ ) and photographed under UV-illumination.

#### II-11. Measurement of topoisomerase activity depending on temperature

The standard assay mixture contained with 200 ng of negatively supercoiled pGEM3zf(-) DNA, 2  $\mu\text{l}$  of 10x reaction buffer (50 mM Tris-HCl, pH 8.0, BSA (1  $\text{mg}/\text{ml}$ ), 1 mM DTT, 2.5 mM EDTA, pH 8.0, and 5 mM  $\text{MgCl}_2$ ), and 0.5  $\mu\text{l}$  of the sample to be tested and adjusted to a final volume of 20  $\mu\text{l}$ . The mixture was incubated for 30 min at various temperature conditions (15, 20, 30, 40, 50, 55, 60 and 70°C). The reaction was terminated by addition of 1  $\mu\text{l}$  of stop reagent (0.5 M EDTA, pH 8.0). Loading buffer was added and sample was run on 1.3% agarose gel without EtBr. After electrophoresis, the gel was stained with EtBr (0.5  $\mu\text{g}/\text{ml}$ ) and photographed under UV-illumination.



## II-12. Measurement of topoisomerase activity depending on various inhibitors

The standard assay mixture contained with 200 ng of negatively supercoiled pGEM3zf(-) DNA, 2  $\mu\text{l}$  of 10x reaction buffer (50 mM Tris-HCl, pH 8.0, BSA (1 mg/ml), 1 mM DTT, 2.5 mM EDTA, pH 8.0, and 5 mM  $\text{MgCl}_2$ ), each inhibitor of various concentrations, such as camptothecin, nalidix acid, spermidine, etoposide and 0.5  $\mu\text{l}$  of the sample to be tested and adjusted to a final volume of 20  $\mu\text{l}$ . The mixture was incubated for 30 min at 37°C. The reaction was terminated by addition of 5  $\mu\text{l}$  of stop reagent (0.5 M EDTA, pH 8.0). Loading buffer was added and sample was run on 1.3% agarose gel without EtBr. After electrophoresis, the gel was stained with EtBr (0.5  $\mu\text{g/ml}$ ) and photographed under UV-illumination.

## II-13. Measurement of topoisomerase activity depending on divalent cations

The standard assay mixture contained with 3  $\mu\text{l}$  of negatively supercoiled pGEM3zf(-) DNA, 2  $\mu\text{l}$  of 10x reaction buffer (50 mM Tris-HCl, pH 8.0, BSA (1 mg/ml), 1 mM DTT, 2.5 mM EDTA, pH 8.0), 4 mM of various divalent ions ( $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$  and

Zn<sup>2+</sup>) and then 0.5  $\mu\text{l}$  of the sample to be tested and adjusted to a final volume of 20  $\mu\text{l}$ . The mixture was incubated for 30 min at 37°C. The reaction was terminated by addition of 5  $\mu\text{l}$  of stop reagent (0.5 M EDTA, pH 8.0). Loading buffer was added and sample was run on 1.3% agarose gel without EtBr. After electrophoresis, the gel was stained with EtBr (0.5  $\mu\text{g}/\text{ml}$ ) and photographed under UV-illumination.

#### II-14. Measurement of topoisomerase activity depending on EDTA, NaCl and ATP

The standard assay mixture contained with 3  $\mu\text{l}$  of negatively supercoiled pGEM3zf(-) DNA, 2  $\mu\text{l}$  of 10x reaction buffer (50 mM Tris-HCl, pH 8.0, BSA (1  $\text{mg}/\text{ml}$ ), 1 mM DTT, 5 mM MgCl<sub>2</sub>), EDTA, NaCl and ATP of various concentrations and 0.5  $\mu\text{l}$  of the sample to be tested and adjusted to a final volume of 20  $\mu\text{l}$ . The mixture was incubated for 30 min at 37°C. The reaction was terminated by addition of 5  $\mu\text{l}$  of stop reagent (0.5 M EDTA, pH 8.0). Loading buffer was added and sample was run on 1.3% agarose gel without EtBr. After electrophoresis, the gel was stained with EtBr (0.5  $\mu\text{g}/\text{ml}$ ) and photographed under UV-illumination.

## II-15. Purification and radiolabelling of oligonucleotides

DNA fragments generated after restriction enzyme digestion with *EcoR* I and were end-filled with either [ $\alpha$ -<sup>32</sup>P]dATP or [ $\gamma$ -<sup>32</sup>P]ATP, using Klenow fragment or T4 polynucleotide kinase. Radiolabeled fragments were purified using Sephadex G-50 spin columns.

## II-16. Electrophoretic mobility shift assay (EMSA)

Non-covalent enzyme-DNA complexes were formed by using 50 mM Tris-HCl (pH 8), 0.5 mM EDTA, with or without 5 mM MgCl<sub>2</sub>, 100 fmol radiolabeled oligonucleotide and 2 – 4 U of purified enzyme by incubation on 37°C for 30 min. The products were separated in an 8% native polyacryamide gel (30: 0.8) using 0.5 X TBE as the running buffer. The gels were electrophoresed at 100 V at room temperature, dried on 3 mm paper and then subjected to autoradiography.

### III. Results and Discussion

#### III-1. PCR cloning of topoisomerase I gene from *Staphylococcus aureus*

To clone the topoisomerase gene I, chromosomal DNA was extracted from *Staphylococcus aureus* sp. strain C-66 cells and amplified the gene by polymerase chain reaction. The amplified DNA fragments were ligated into the *Xho* I/*Kpn* I-cleaved pBAD/His A expression vectors with T4 DNA ligase. The ligated DNA was transformed and expressed in *E. coli* strain Top 10. Fig. 1 shows the physical map of recombinant pTP I plasmid. The 6.1 kb recombinant plasmid pTP I containing 2,070 bp insert and pBAD/His A expression vector (Fig. 2). The nucleotide sequence of the cloned topoisomerase I gene was translated to amino acid and the deduced sequence was compared with those of other *Staphylococcus aureus*. Overall amino acid sequence conservation among topoisomerases I could be seen in the amino acid alignment. As shown in Fig. 3, 99% of the amino acid sequence was conserved in the alignment.

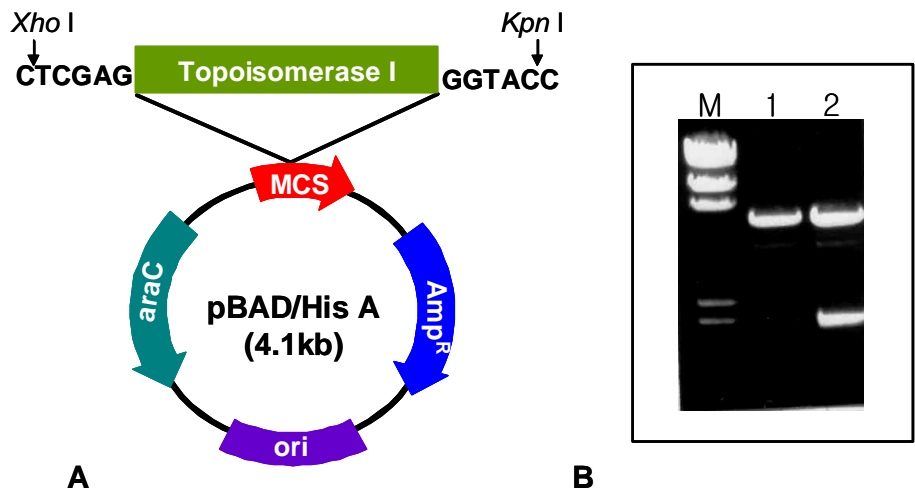


Figure 1. Construction of a pTP I. A: physical map of pTP I. B: Restriction digest of pTP1. Lane M,  $\lambda$  /Hind III cut marker; Lane 1, pBAD/His A vector digested with *Xho*I and *Kpn*I; Lane 2, pTP I digested with *Xho*I and *Kpn*I.

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TTGGCAGATAATTTAGTCATTGTTGAATCGCCTGCAAAAGCAAAAACCATTGAAAAGTAT 60
M A D N L V I V E S P A K A K T I E K Y
TTAGGTAAGAAATATAAAGTTATAGCTTCAATGGGACACGTCAGAGACTTACCAAGAAGT 120
L G K K Y K V I A S M G H V R D L P R S
CAAATGGGTGTCGACACTGAAGATAATTACGAACCAAAATATATAACAATACGCGGAAAA 180
Q M G V D T E D N Y E P K Y I T I R G K
GGTCCTGTTGTAAAAGAATTGAAAAAACATGCAAAAAAAGCGAAAAACGTCTTTCTCGCA 240
G P V V K E L K K H A K K A K N V F L A
AGTGACCCCGACCGTGAAGGTGAAGCAATTGCTTGGCATTATCAAAAATTTTAGAGCTT 300
S D P D R E G E A I A W H L S K I L E L
GAAGATTCTAAAGAAAATCGCGTTGTTTTCAACGAAATAACTAAAGACGCTGTTAAAGAA 360
E D S K E N R V V F N E I T K D A V K E
AGTTTTAAAAATCCTAGAGAAATTGAAATGAACTTAGTCGATGCACAACAAGCGCGTCGA 420
S F K N P R E I E M N L V D A Q Q A R R
ATATTAGATAGATTGGTTGGCTATAACATCTCGCCAGTTCTATGAAAAAAGTGAAAAAA 480
I L D R L V G Y N I S P V L W K K V K K
GGGTTGTCAGCGGGTCGAGTTCAATCTGTGCACTTCGTTTAGTCATTGACCGTGAAAAAT 540
G L S A G R V Q S V A L R L V I D R E N
GAAATACGAAACTTTAAACCAGAAGAATATTGGACTATTGAAGGAGAATTTAGATACAAA 600
E I R N F K P E E Y W T I E G E F R Y K
AAATCAAAATTCAATGCTAAATTCCTTCATTATAAAAAATAAACCTTTTAAATTAAAAACG 660
K S K F N A K F L H Y K N K P F K L K T
AAAAAAGATGTTGAGAAAATTACAACTGCATTAGATGGAGATCAATTGAAATTACAAAC 720
K K D V E K I T T A L D G D Q F E I T N
GTGACTAAAAAAGAAAAACGCGTAATCCAGCAAACCCATTTACAACCTCTACATTACAA 780
V T K K E K T R N P A N P F T T S T L Q
CAAGAGGCGGCACGTAAATTAAACTTTAAAGCTAGAAAAACAATGATGGTCGCACAACAA 840
Q E A A R K L N F K A R K T M M V A Q Q
TTATATGAAGGTATAGATTTGAAAAACAAGGTACGATTGGTTTAATAACATATATGAGA 900
L Y E G I D L K K Q G T I G L I T Y M R
ACCGATTCTACACGTATTTTCAGATACTGCCAAAGCTGAAGCAAAACAGTATATAACTAAT 960
T D S T R I S D T A K A E A K Q Y I T N
AAATACGGTGAATCTTACACTTCTAAACGTAAAGCATCAGGGAAACAAGGTGACCAAGAT 1020
K Y G E S Y T S K R K A S G K Q G D Q D
GCCCATGAGGCTATTAGACCTTCAAGTACTATGCGTACGCCAGATGATATGAAGTCATTT 1080
A H E A I R P S S T M R T P D D M K S F

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To be continued,

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TTACGAAAGACCAATACCGATTATACAAATTAATTTGGAACGATTGTGCTAGTCAA 1140
L T K D Q Y R L Y K L I W E R F V A S Q
ATGGCTCCAGCAATACTTGATACAGTCTCATTAGACATAACACAAGGTGACATTAAATTT 1200
M A P A I L D T V S L D I T Q G D I K F
AGAGCGAATGGTCAAACAATCAAGTTCAAAGGATTTATGACACTTTATGTAGAACTAAA 1260
R A N G Q T I K F K G F M T L Y V E T K
GATGATAGTGATAGCGAAAAGGAAAATAAACTGCCTAAATTAGAGCAAGGTGATAAAGTC 1320
D D S D S E K E N K L P K L E Q G D K V
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T A T Q I E P A Q H Y T Q P P P R Y T E
GCGAGATTAGTAAAAACACTAGAAGAATTGAAAATTGGGCGACCATCAACTTATGCACCG 1440
A R L V K T L E E L K I G R P S T Y A P
ACAATAGATACGATTCAAAGCGTAACCTATGTCAAATTAGAAAGTAAGCGTTTGTTCCT 1500
T I D T I Q K R N Y V K L E S K R F V P
ACTGAGTTGGGAGAAATAGTTTCATGAACAAGTGAAAGAATACTTCCCAGAGATTATTGAT 1560
T E L G E I V H E Q V K E Y F P E I I D
GTGGAATTCACAGTGAATATGGAACGTTACTTGATAAGATTGCAGAAGGCGACATTACA 1620
V E F T V N M E T L L D K I A E G D I T
TGGAGAAAAGTAATCGACGGTTTCTTTAGTAGCTTTAAACAAGATGTTGAACGTGCTGAA 1680
W R K V I D G F F S S F K Q D V E R A E
GAAGAGATGGAAAAGATTGAAATCAAAGATGAGCCAGCCGGTGAAGACTGTGAAGTTTGT 1740
E E M E K I E I K D E P A G E D C E V C
GGTTCCTCTATGGTTATAAAAAATGGGCGCTATGGTAAGTTTATGGCTTGCTCAAACCTC 1800
G S P M V I K M G R Y G K F M A C S N F
CCGGATTGTCTGTAATACAAAAGCGATAGTTAAGTCTATTGGTGTTAAATGTCCAAAATGT 1860
P D C R N T K A I V K S I G V K C P K C
AATGATGGTGACGTCGTAGAAAAGAAAATCTAAAAAGAATCGTGTCTTTTATGGATGTTTCG 1920
N D G D V V E R K S K K N R V F Y G C S
AAATATCCTGAATGCGACTTTATCTCTTGGGATAAGCCGATTGGAAGAGATTGTCCAAAA 1980
K Y P E C D F I S W D K P I G R D C P K
TGTAACCAATATCTTGTGAAAAATAAAAAAGGCAAGACAACACAAGTAATATGTTCAAAT 2040
C N Q Y L V E N K K G K T T Q V I C S N
TGCGATTATAAAGAGGCAGCGCAGAAATAA 2070
C D Y K E A A Q K #

```

**Figure 2.** DNA sequence of a topoisomerase I gene from *Staphylococcus aureus*

MRSA252	LADNLVIVESPAKAKTIEKYLGGKKYVIASMGHVRDLPRSQMGVDTEDNYEPKYITIRGK	60	MRSA252	LTQDQYRLYKLIWERFVASQMAPAILDTVSLDITQGDIKFRANGQTIKFGFMFLYVETK	420
COL	LADNLVIVESPAKAKTIEKYLGGKKYVIASMGHVRDLPRSQMGVDTEDNYEPKYITIRGK	60	COL	LTQDQYRLYKLIWERFVASQMAPAILDTVSLDITQGDIKFRANGQTIKFGFMFLYVETK	420
C-66	LADNLVIVESPAKAKTIEKYLGGKKYVIASMGHVRDLPRSQMGVDTEDNYEPKYITIRGK	60	C-66	LTQDQYRLYKLIWERFVASQMAPAILDTVSLDITQGDIKFRANGQTIKFGFMFLYVETK	420
MSSA476	LADNLVIVESPAKAKTIEKYLGGKKYVIASMGHVRDLPRSQMGVDTEDNYEPKYITIRGK	60	MSSA476	LTQDQYRLYKLIWERFVASQMAPAILDTVSLDITQGDIKFRANGQTIKFGFMFLYVETK	420
	*****			*****	
MRSA252	GPVVKELKKHAKKAKNVFLASDPDREGAIAWHLKSILELEDSKENRVVFNEITKDAVKE	120	MRSA252	DDSDSEKENKLPKLEQGDKVTATQIEPAQHYTQPPRYTEARLVKTLLEELKIGRPSTYAP	480
COL	GPVVKELKKHAKKAKNVFLASDPDREGAIAWHLKSILELEDSKENRVVFNEITKDAVKE	120	COL	DDSDSEKENKLPKLEQGDKVTATQIEPAQHYTQPPRYTEARLVKTLLEELKIGRPSTYAP	480
C-66	GPVVKELKKHAKKAKNVFLASDPDREGAIAWHLKSILELEDSKENRVVFNEITKDAVKE	120	C-66	DDSDSEKENKLPKLEQGDKVTATQIEPAQHYTQPPRYTEARLVKTLLEELKIGRPSTYAP	480
MSSA476	GPVVKELKKHAKKAKNVFLASDPDREGAIAWHLKSILELEDSKENRVVFNEITKDAVKE	120	MSSA476	DDSDSEKENKLPKLEQGDKVTATQIEPAQHYTQPPRYTEARLVKTLLEELKIGRPSTYAP	480
	*****			*****	
MRSA252	SFKNPREIEMNLVDAQARRILDRLVGYNISPVLWKKVKKLSAGRVQSVALRLVIDREN	180	MRSA252	TIDTIQKRNYVKLESKRFPVPTLGEIVHEQVKEYFPEIIDVEFTVNMETLLDKIAEGDIT	540
COL	SFKNPREIEMNLVDAQARRILDRLVGYNISPVLWKKVKKLSAGRVQSVALRLVIDREN	180	COL	TIDTIQKRNYVKLESKRFPVPTLGEIVHEQVKEYFPEIIDVEFTVNMETLLDKIAEGDIT	540
C-66	SFKNPREIEMNLVDAQARRILDRLVGYNISPVLWKKVKKLSAGRVQSVALRLVIDREN	180	C-66	TIDTIQKRNYVKLESKRFPVPTLGEIVHEQVKEYFPEIIDVEFTVNMETLLDKIAEGDIT	540
MSSA476	SFKNPREIEMNLVDAQARRILDRLVGYNISPVLWKKVKKLSAGRVQSVALRLVIDREN	180	MSSA476	TIDTIQKRNYVKLESKRFPVPTLGEIVHEQVKEYFPEIIDVEFTVNMETLLDKIAEGDIT	540
	*****			*****	
MRSA252	EIRNFKPEEYWTIEGEFRYKSKFNAPFLHYKNKPFLLTKKDVKEITIALDGDQFEITN	240	MRSA252	WRKVIDGFFSSFKQDVERAEEEMEKIEIKDEPAGEDCHVCGSPMWIKMGRYKGFMACSNF	600
COL	EIRNFKPEEYWTIEGEFRYKSKFNAPFLHYKNKPFLLTKKDVKEITIALDGDQFEITN	240	COL	WRKVIDGFFSSFKQDVERAEEEMEKIEIKDEPAGEDCHVCGSPMWIKMGRYKGFMACSNF	600
C-66	EIRNFKPEEYWTIEGEFRYKSKFNAPFLHYKNKPFLLTKKDVKEITIALDGDQFEITN	240	C-66	WRKVIDGFFSSFKQDVERAEEEMEKIEIKDEPAGEDCHVCGSPMWIKMGRYKGFMACSNF	600
MSSA476	EIRNFKPEEYWTIEGEFRYKSKFNAPFLHYKNKPFLLTKKDVKEITIALDGDQFEITN	240	MSSA476	WRKVIDGFFSSFKQDVERAEEEMEKIEIKDEPAGEDCHVCGSPMWIKMGRYKGFMACSNF	600
	*****			*****	
MRSA252	VTKKEKTRNPANPFTTSTLQOEAAARKLNFKARKTMMVAQQLYEGIDLKKQGTIGLITYMR	300	MRSA252	PDCRNTKAIVKSIGVKCPKCNDDGVVERKSKKNRVFYGCSKYPECDFISWDKPIGRDCPK	660
COL	VTKKEKTRNPANPFTTSTLQOEAAARKLNFKARKTMMVAQQLYEGIDLKKQGTIGLITYMR	300	COL	PDCRNTKAIVKSIGVKCPKCNDDGVVERKSKKNRVFYGCSKYPECDFISWDKPIGRDCPK	660
C-66	VTKKEKTRNPANPFTTSTLQOEAAARKLNFKARKTMMVAQQLYEGIDLKKQGTIGLITYMR	300	C-66	PDCRNTKAIVKSIGVKCPKCNDDGVVERKSKKNRVFYGCSKYPECDFISWDKPIGRDCPK	660
MSSA476	VTKKEKTRNPANPFTTSTLQOEAAARKLNFKARKTMMVAQQLYEGIDLKKQGTIGLITYMR	300	MSSA476	PDCRNTKAIVKSIGVKCPKCNDDGVVERKSKKNRVFYGCSKYPECDFISWDKPIGRDCPK	660
	*****			*****	
MRSA252	TDSTRISDTAKALAKQYITDYGESYTSKRKASGKGQDQDAHEAIRPSSMTPTDDMKSF	360	MRSA252	CNQYLVENKKGKTTQVICSNC DYKEAAQK	689
COL	TDSTRISDTAKALAKQYITDYGESYTSKRKASGKGQDQDAHEAIRPSSMTPTDDMKSF	360	COL	CNQYLVENKKGKTTQVICSNC DYKEAAQK	689
C-66	TDSTRISDTAKALAKQYITDYGESYTSKRKASGKGQDQDAHEAIRPSSMTPTDDMKSF	360	C-66	CNQYLVENKKGKTTQVICSNC DYKEAAQK	689
MSSA476	TDSTRISDTAKALAKQYITDYGESYTSKRKASGKGQDQDAHEAIRPSSMTPTDDMKSF	360	MSSA476	CNQYLVENKKGKTTQVICSNC DYKEAAQK	689
	*****			*****	

Figure 3. Alignment of predicted amino acid sequence encoded by topoisomerase I genes of other *Staphylococcus aureus* strains



### III-2. Purification of a topoisomerase I from *E. coli* cells harboring pTP I

To purify the topoisomerase from *E. coli* cells harboring pTP I, the cultured cells were harvested and sonicated. Upon induction with 0.02% L-arabinose, *E. coli* strain Top 10 cells harboring the recombinant plasmid overproduced a polypeptide with an estimated molecular mass of 79.1 kDa, a size similar to that expected for the putative *Staphylococcus aureus* topoisomerase I, respectively.

To obtain the *Staphylococcus aureus* topoisomerase I, ammonium sulfate fractionation and affinity chromatography on Hitrap chelating column were performed in order. Most of type I topoisomerase activities were recovered from crude cell extract between 30 and 70% saturation of ammonium sulfate. The ammonium sulfate fractionated proteins were subjected to PD-10 column to remove salt and the proteins were concentrated as described in Materials and Methods.

The concentrated proteins were separated by Hitrap chelating chromatography. The Hitrap chelating chromatography resulted in the elimination of the majority of non-specific proteins with good recovery of topoisomerase. There was no nonspecific nuclease activity. As shown in Fig. 4, the topoisomerase activity was recovered at 0.1 M imidazole and formed a broad trailing peak up to 0.25 M imidazole. The highest activity was found around 0.2 M

imidazol.

The DNA relaxation assay was used to monitor the enzyme activity at a step of purification (Fig. 4). The purified enzyme was serially diluted and incubated with native pTP I plasmid under the standard assay conditions. As shown in Fig. 4, the purified topoisomerase I activity emerged in the fractions from 10 to 17. Protein fractions were separated on a 12% SDS–polyacrylamide gel. The major band was correlated with the relaxation activity of plasmid DNA, as shown in Fig. 2. Total activity of the enzyme was 9,900 units (Table 2).

The purified enzyme showed a typical relaxation activity on covalently closed circular DNA (form I). In standard reaction condition, the relaxation activity occurred rapidly on added substrate DNA (form I) and then the substrate DNA (form I) relaxed to open circular DNA (form II). These results suggest that the purified enzyme has an efficient relaxation activity on negatively supercoiled DNA.

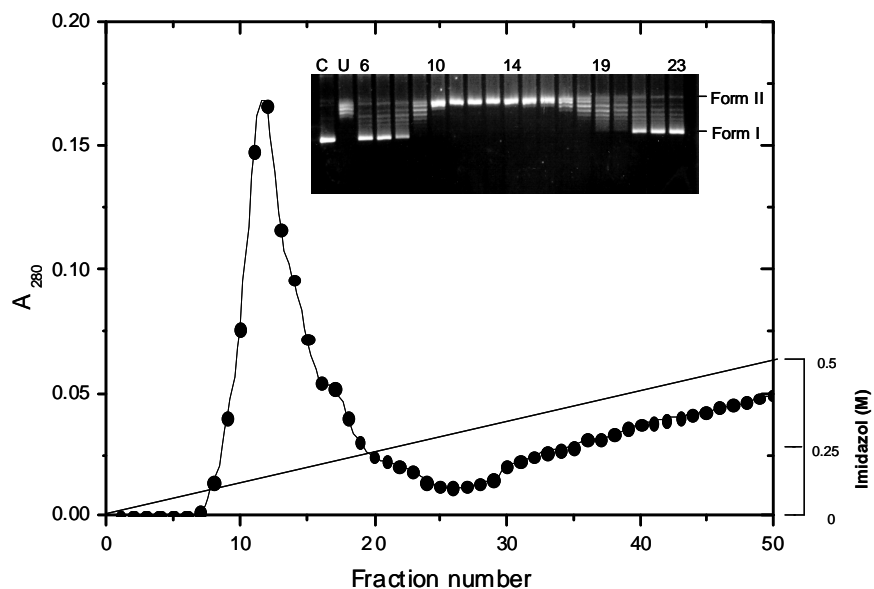


Figure 4. FPLC elution profile of the Hitrap chelating column. Typical topoisomerase activity assay with each fraction was performed in a standard reaction mixture and products were visualized on 1.3% agarose gel.

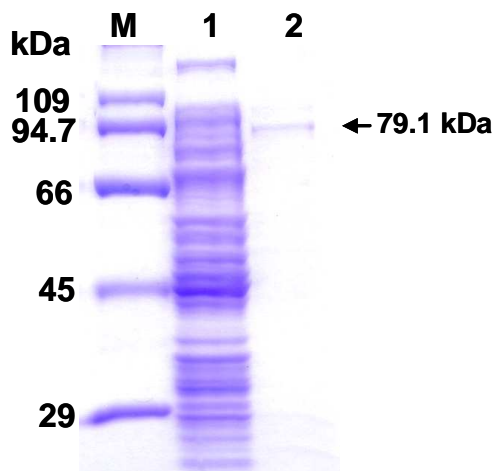


Figure 5. SDS-PAGE analysis of protein purification of topoisomerase I from *Staphylococcus aureus* sp. strain C-66. Lane M, protein size marker; lane 1, crude cell extract; lane 2, Hitrap chelating column.

**Table 3. Summary of purification of topoisomerase I from *E. coli* Top 10**

<b>Fraction</b>	<b>Total protein (mg)</b>	<b>Total activity (U)<sup>a</sup></b>	<b>Specific activity (U/mg)</b>	<b>Yield (%)</b>
<b>Cell extract</b>	375.64	ND <sup>b</sup>	ND <sup>b</sup>	ND <sup>b</sup>
<b>(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub><sup>c</sup></b>	170.61	128,242	754	100
<b>Hitrap chelating column</b>	1.98	9,900	5,000	7.7

a. One unit topoisomerase activity was defined as the amount of enzyme required to relax 50% of supercoiled pGEM3zf(–) DNA (200 ng) for 30 min at 37°C in the presence of 5 mM of MgCl<sub>2</sub>.

b. ND, not detectable

c. The range of saturation concentration for the fractionation was 20~70%.

### III-3. Effect of time, pH and temperature on purified topoisomerase activity

To check the effects of various times, pHs and temperatures on purified topoisomerase activity were investigated (Figs. 4, 5A and 5B). Standard assay mixtures were incubated by the addition of the enzyme at different time. As shown in Fig. 4, the relaxation activity of the purified enzyme was exerted its catalytic activity after 10 min and was activated at 30 min after adding the DNA substrates (Fig. 4). These results indicate that the purified topoisomerase possesses the relaxation activity, especially after 30 min. The relaxation activity was determined by various range of pH and temperature. As shown in Fig. 5A, the enzyme was active especially in pH 7.5. The enzyme also was active at 37~40°C of temperature and the optimal temperature was found to be approximately 37°C.

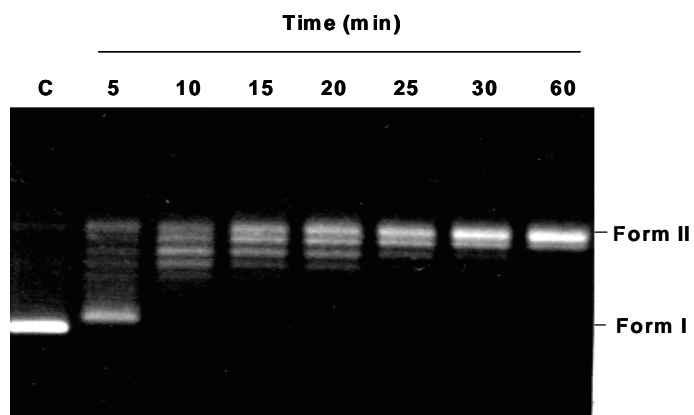


Figure 6. Effect of incubation time on the enzyme activity of the purified topoisomerase. The enzyme activity was assayed in the standard reaction mixture.

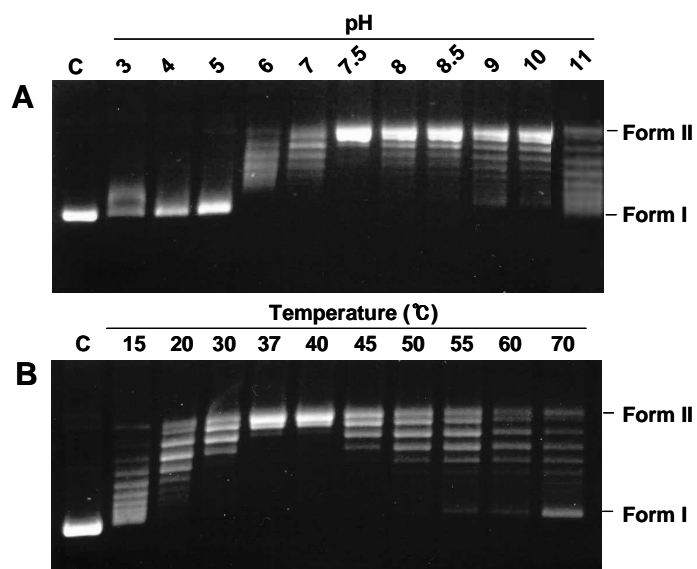


Figure 7. Effect of pH and temperature on the relaxation activity of the purified topoisomerase. The enzyme activity was assayed in the standard reaction mixture under different experiment conditions.



#### III-4. Effect of relaxation activity depending on divalent cations

It is known that most of prokaryotic DNA topoisomerases absolutely require divalent cations for their activities (Bouthier, 1993). Fig. 6 shows the effect of various divalent cations ( $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$  and  $Zn^{2+}$ ) on DNA relaxation activity of the purified enzyme. The enzyme was inactive in the absence of a divalent cation.  $Mg^{2+}$ ,  $Mn^{2+}$  and  $Ca^{2+}$  were able to support the DNA relaxation activity of the enzyme, whereas  $Cu^{2+}$  and  $Zn^{2+}$  cations were inhibitory.  $Mg^{2+}$  was the most preferred ion for the purified enzyme, as all prokaryotic type I DNA topoisomerases. Especially, in the case of purified enzyme, the relaxation activity is strongly activated by  $Mg^{2+}$ ,  $Mn^{2+}$  and  $Cu^{2+}$ , whereas there was no effect by  $Ca^{2+}$  and  $Zn^{2+}$ . The results indicate that a divalent cation, especially  $Mg^{2+}$  cation, is indispensable for the relaxation activity of a type I topoisomerase.

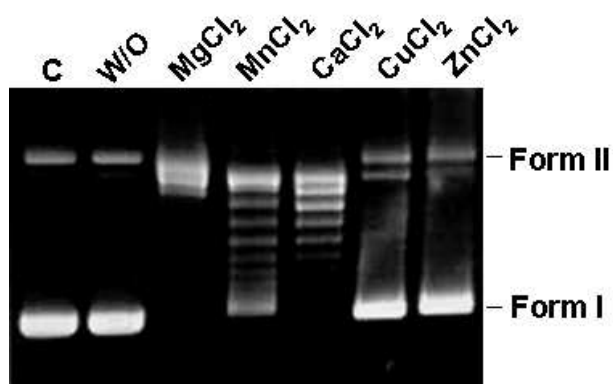


Figure 8. Effect of divalent cations on the relaxation activity of purified topoisomerase. Various kinds of 4 mM divalent cations were added to the standard reaction mixture and the standard reaction mixture was incubated for 30 min at 37°C. The reaction products were analyzed on 1.3% agarose gel.

### III-5. Effect of relaxation activity depending on EDTA, NaCl and ATP

The effect of some inhibitors on topoisomerase I activity are shown in Fig. 7. The purified topoisomerase activity was inhibited by EDTA. As shown in Fig. 7A, it activated the relaxation activity at a lower concentration of EDTA, but strongly inhibited the relaxation activity of topoisomerase I at a higher concentration (5 and 10 mM) of EDTA.

The effect of NaCl on the relaxation activity of *Staphylococcus aureus* topoisomerase I is presented in Fig. 7B. The enzyme shows a predominantly processive mode in the absence of NaCl and gradually shifts to a distributive mode with the increase in the NaCl concentration. The results shows that there is an increase in the number of partially relaxed DNA, which reflects a distributive mode of action by the enzyme. However, as the salt concentration increased, the enzyme appeared to have switched to a distributive mode in which all of the input DNA molecules were relaxed to a similar but progressively smaller extent. The similar switch in the mode of action in response to the change in salt concentration was described previously for *M. smegatis* topoisomerase I (Bhaduri , 1998). It is possible that high concentrations of NaCl reduce the affinity of a topoisomerase for DNA, facilitating the dissociation of the enzyme from the substrate before the completion of the relaxation reaction.

The type of topoisomerase for the purified enzyme was also determined on the basis of ATP-requirement. As shown in Fig. 7C,

the purified topoisomerase did not require ATP for the relaxation activity. The substrate DNA was relaxed by the purified topoisomerase I in the absence or presence of ATP. These results indicate that the purified enzyme is a topoisomerase I which possess ATP-independent manner.

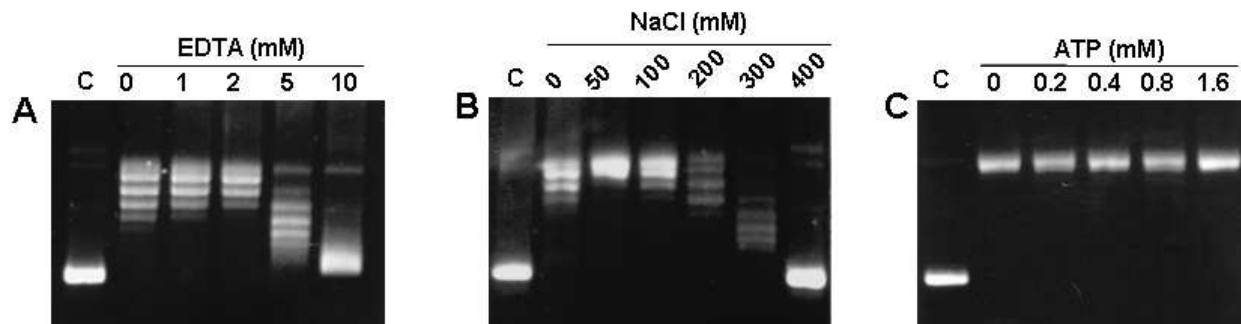


Figure 9. Effect of EDTA, NaCl and ATP on the relaxation activity of purified topoisomerase. Enzyme activity was assayed in standard reaction mixture containing either different concentrations of EDTA (A), NaCl (B) and ATP (C).

### III-6. Effect of relaxation activity depending on various inhibitors

To determine whether the enzyme belongs to type I or type II DNA topoisomerase, the effects of various inhibitors on the purified enzyme, such as camptothecin, nalidixic acid, spermidine, and etoposide were examined. Camptothecin and nalidixic acid were used as type I- and type II-specific inhibitors respectively (Morris and Geller, 1996; Desai, 1997; Alkorta, 1999; Carlos, 2000; Miller and Niell, 2001). Camptothecin stimulates DNA single strand breaks by preferentially trapping a subset of the topoisomerase I cleavage sites (Jaxel, 1991; Pommier, 1995; Kjeldsen, 1988). It has been established that camptothecin inhibits specifically topoisomerase I by stabilizing the cleavable complexes, which are enzyme-catalyzed DNA single-strand breaks with the enzyme linked to the 3'-terminus of the break (Fukasawa, 1998). As shown in Figure 8, the enzyme activity was completely inhibited by treatment with 0.2 mM camptothecin but not by nalidixic acid. These results suggest that the purified topoisomerase belongs to type I topoisomerase.

However, as shown in Fig. 8, topoisomerase II-specific inhibitors, such as nalidixic acid, etoposide, and spermidine had no inhibitory effect on the purified topoisomerase. Taken together, the results suggest that the purified topoisomerase from *Staphylococcus aureus* sp. strain C-66 belongs to the prokaryotic DNA topoisomerase I.

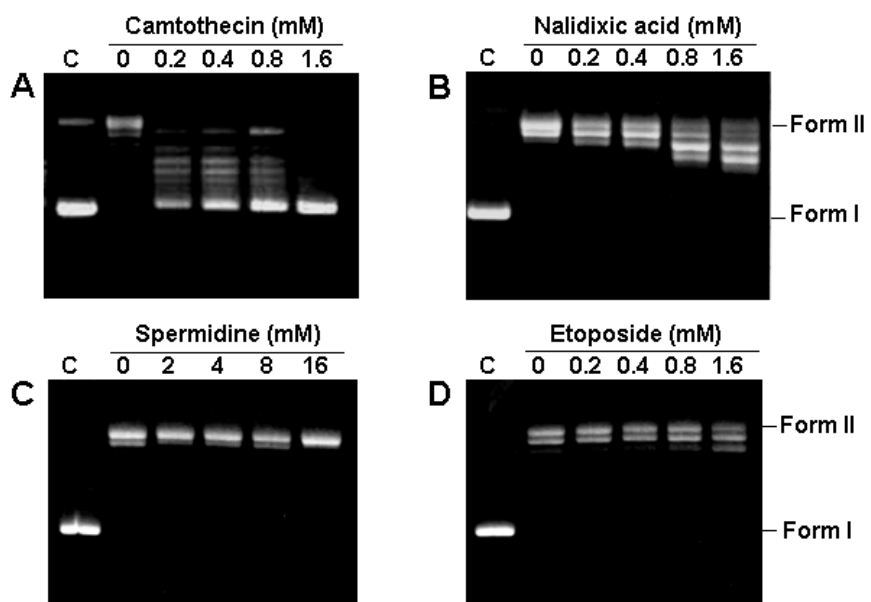


Figure 10. Effect of topoisomerase-specific inhibitors on the relaxation activity of purified topoisomerase. (A) Camptothecin; (B) Nalidixic acid; (C) Spermidine; (D) Etoposide. Various concentrations of inhibitors were added to the standard reaction mixture as indicated and incubated for 30 min at 37°C.

### III-7. Analysis of the reaction intermediate

The covalent protein DNA intermediate complex formation described above could be mediated either by the 3' or 5'-end of nicked DNA. The experimental strategy to determine the linkage of *S. aureus* enzyme is shown in Fig. 9. This experiment is based on the observation that covalent protein-DNA complex migrates more slowly than free DNA molecule when it subjected to 8% native polyacrylamide gel electrophoresis. Determination of the linkage could be established by employing DNA substrates labeled at 3'-end. Covalent protein DNA intermediate complex was migrate more slowly than free DNA molecules when the 3'-end labeled DNA substrates were used. When 5'-end labeled DNA substrates were used, no appreciable complex formation was observed (Fig. 9). These results demonstrate that covalent complex formation is mediated through the 5'-end of the nicked DNA.



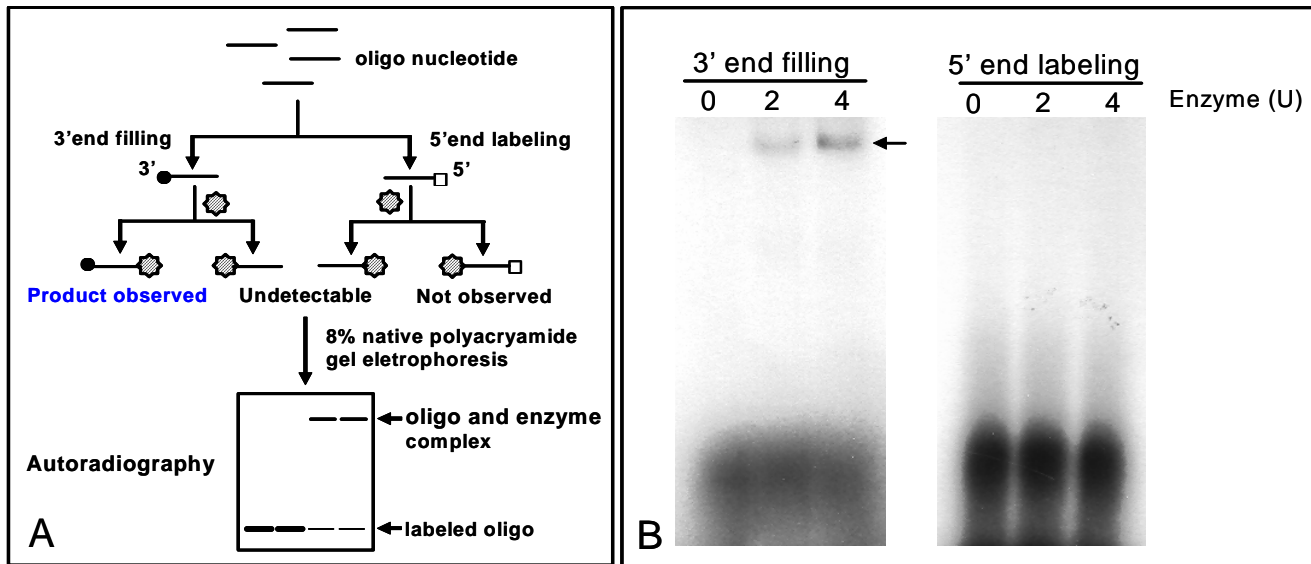


Figure 11. The enzyme makes covalent linkage with the 5'-end of the nick. A, the experimental strategy. B, 5' and 3' end labeled fragment were complexed with protein. The complexes were electrophoresed on 8% native polyacrylamide gel followed by electrophoretic mobility shift assay.

### III-8. Comparison of the relaxation activity with *S. aureus* topoisomerase I and *Methylophaga* topoisomerase I

In this study, the purified topoisomerase I was characterized at various conditions. These results predict that the purified enzyme is an ATP-independent topoisomerase I. And the relaxation activity of the purified enzyme was compared with *Methylophaga* topoisomerase I. The relaxation activity of both *S. aureus* and *Methylophaga* topoisomerase I showed the typical prokaryotic DNA topoisomerase I. Nevertheless, There is a few difference between two enzymes. As shown in Table 4, The purified topoisomerase observed that is a specific topoisomerase possessing a very strong activity in treatment of NaCl, EDTA and camptothecin compared with *Methylophaga* topoisomerase I. And the purified enzyme has no activity in the presence  $\text{Cu}^{2+}$ . As a result, comparison of the relaxation activity with *Methylophaga* topoisomerase suggest that the purified enzyme is a typical prokaryotic topoisomerase I.

Table 4. Comparison of the relaxation activity with *S. aureus* topoisomerase I and *Methylophaga* topoisomerase I

Property		Topoisomerase I	
		<i>S. aureus</i>	<i>Methylophaga</i>
Cofactor requirement for relaxation activity	Mg <sup>2+</sup>	+	+
	Mn <sup>2+</sup>	+	+
	Ca <sup>2+</sup>	+	Very low
	Cu <sup>2+</sup>	–	+
	Zn <sup>2+</sup>	–	–
	ATP	–	–
Sensitivity to inhibitors	Camptothecin	+	+
	Nalidixic acid	Very low	–
	Spermidine	–	Very low
	Etoposide	–	–
	NaCl	≥ 100 mM	≥ 1 mM
	EDTA	≥ 5 mM	≥ 0.5 mM

## V. 적 요

### 포도상구균으로부터 DNA topoisomerase I 유전자의 클로닝 및 특성에 관한 연구

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본 연구에서 type I DNA topoisomerase 유전자를 황색포도상구균 균주 C-66 세포로부터 염색체 DNA를 분리하고 중합연쇄반응을 통하여 그 유전자를 증폭시킨 후 pBAD/His A 발현벡터에 클로닝하고 대장균 Top 10 세포에서 발현을 유도하였다. 정제한 topoisomerase의 유전자는 630개의 아미노산을 암호화할 수 있는 2,070개의 뉴클레오타이드로 구성되어 있었다. 0.02%의 L-arabinose를 처리하여 topoisomerase I의 발현을 유도한 후 Hitrap chelating column을 이용하여 정제하였다. 분리한 효소는 37℃와 pH 7.5에서 가장 높은 활성을 보였고, 효소의 활성에  $Mg^{2+}$  이온이 필요함을 관찰하였다. 또한 EDTA와 NaCl, ATP에 대한 영향을 관찰한 결과, 10 mM의 EDTA, 400 mM의 NaCl 있을 때 효소의 활성이 완전히 사라짐을 관찰하였다.

정제된 효소의 특성을 규명하기 위하여 다양한 topoisomerase 억제제를 이용하여 효소 활성을 측정한 결과 type I DNA topoisomerase의 억제제인 camptothecin을 처리하였을 때 효소의 활성은 억제되지만 type II topoisomerase 억제제인 nalidixic acid, etoposide 그리고 spermidine을 처리하였을 경우, 그 활성은 억제되지 않았다. 또한 정제된 효소는 5'

끝 부위를 절단하여 효소의 기작이 시작됨을 electrophoretic mobility shift assay를 통하여 증명하였다.

이상의 결과는 정제된 topoisomerase는 전형적인 ATP 비의존적 type I DNA topoisomerase임을 제시하는 것이다.

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