Bacillus cereus DNA topoisomerase I and IIIα: purification, characterization and complementation of *Escherichia coli* TopoIII activity

Zhiyu Li, Hiroshi Hiasa[1] and Russell DiGate*

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, MA, USA and
[1]Department of Pharmacology, University of Minnesota Medical School, MN, USA

ABSTRACT

The *Bacillus cereus* genome possesses three type IA topoisomerase genes. These genes, encoding DNA topoisomerase I and IIIα (*bc*Topo I, *bc*Topo IIIα), have been cloned into T7 RNA polymerase-regulated plasmid expression vectors and the enzymes have been overexpressed, purified and characterized. The proteins exhibit similar biochemical activity to their *Escherichia coli* counterparts, DNA topoisomerase I and III (*ec*Topo I, *ec*Topo III). *bc*Topo I is capable of efficiently relaxing negatively supercoiled DNA in the presence of Mg^{2+} but does not possess an efficient DNA decatenation activity. *bc*Topo IIIα is an active topoisomerase that is capable of relaxing supercoiled DNA at a broad range of Mg^{2+} concentrations; however, its DNA relaxation activity is not as efficient as that of *bc*Topo I. In addition, *bc*Topo III is a potent DNA decatenase that resolves *oriC*-based plasmid replication intermediates *in vitro*. Interestingly, *bc*Topo I and *bc*Topo IIIα are both able to compensate for the loss of *ec*Topo III in *E.coli* cells that lack *ec*Topo I. In contrast, *ec*Topo I cannot substitute for *ec*Topo III under these conditions.

INTRODUCTION

DNA topoisomerases resolve entangled DNA intermediates by transiently cleaving one or two DNA strands and passing another intact strand(s) through the nick. Subsequently, the enzymes rejoin the DNA break and finish one round of this catalytic reaction (1,2). Based on their catalytic mechanism, topoisomerases have been categorized into four subfamilies, type IA and IB, and type IIA and IIB. Type IA topoisomerases transiently break single-stranded DNA and form a covalent tyrosyl-DNA phosphodiester bond through a 5′ phosphate. They are highly conserved from bacteria to humans. In most cells, two type IA topoisomerases are present and at least one is needed for viability (3). *Escherichia coli* possesses two type IA topoisomerases, *ec*Topo I (4) and *ec*Topo III (5). Among all characterized type IA topoisomerases, *ec*Topo I-like enzymes are capable of relaxing negatively supercoiled DNA efficiently. Therefore, *ec*Topo I in conjunction with DNA gyrase (a type IIA enzyme) functions in the regulation of chromosomal superhelicity (6,7). Although *ec*Topo III relaxes negatively supercoiled DNA, it is dramatically more efficient in the resolution of interlinked DNA dimers, a process of intermolecular strand passage (8,9). *In vivo*, *topB* (gene encoding *ec*Topo III) null strains exhibit heightened frequencies of illegitimate recombination (10).

Drosophila, mouse and human also have two type IA topoisomerases, topoisomerase 3α and 3β (TOP3α, TOP3β) (11–13). The deletion of the gene encoding mouse TOP3α results in an embryonic lethal phenotype (14), whereas inactivation of TOPβ leads to infertility and a shortened life span (15,16). *Saccharomyces cerevisiae* and *Saccharomyces pombe* only encode a single type IA topoisomerase, Topoisomerase 3 (TOP3). A TOP3 mutation in *S.cerevisiae* causes slow growth, sporulation defects and severe defects in DNA repair and recombination (17). Interestingly, TOP3 in *S.pombe* is essential for survival (18).

A BlastP (19) search of *ec*Topo III homologs in the NCBI microbial genomic database reveals that many bacteria contain two or more chromosomally encoded type IA topoisomerases. Among the *Bacillus*, *Bacillus anthracis* (20), *Bacillus cereus* (21) and *Bacillus thuringiensis* have three chromosomal copies of type IA topoisomerases. Two promiscuous plasmids, pXO1 and pXO2, harbored by *B.anthracis* encode virulence

factors that cause anthrax (22). These plasmids can be mobilized and it has been reported that *B.cereus*, containing pXO1 and pXO2-like plasmids, is able to cause an illness resembling inhalation anthrax in mouse (23). Interestingly, pXO1 and pXO2 appear to encode two additional type IA topoisomerases. Therefore, five type IA topoisomerases may be present in these bacteria. In addition, Gram-positive bacteria that are of clinical importance, such as *Clostoridium pertringens* (24), *Clostoridium tetani* (25), *Enterococcus faecalis* (26) and *Staphylococcus epidermidis* (27), also possess multiple chromosomally-encoded type IA topoisomerases.

Similar to pXO1 and pXO2 of *B.anthracis*, many broad-host-range promiscuous plasmids also encode type IA topoisomerases. For example, the *traE* gene of the IncPα plasmid RP4 encodes a type IA topoisomerase (28). Since these plasmids are also able to mobilize among cells, many bacteria may possess three or more type IA topoisomerases. The biological relevance of multiple copies of type IA topoisomerases, however, remains unclear.

B.cereus is a soil-dwelling opportunistic pathogen that causes infections such as endophthalmitis, bacteremia, septicemia, endocarditis, salpingitis, cutaneous infections, pneumonia and meningitis (23). Since *B.cereus* and *B.anthracis* are closely related and share high degree of genomic sequence identity, the study of the DNA recombination and repair in *B.cereus* may provide valuable information in the understanding of the process of DNA metabolism in *B.anthracis* and help to identify novel targets for antibiotic design and screening. In this study, two of *B.cereus* type IA topoisomerases, annotated *ec*Topo I and *ec*Topo IIIα, have been cloned, expressed, purified and characterized. Although these two enzymes exhibit distinctly different biochemical activity, they both are able to substitute for the loss of *ec*Topo III *in vivo*.

MATERIALS AND METHODS

DNA and nucleotides

ΦX174 replicative form I DNA (covalently closed, negatively supercoiled double stranded DNA) was purchased from Invitrogen. DNA oligonucleotides were prepared by the University of Maryland Biopolymer Laboratory. Radiolabeled nucleoside triphosphate was purchased from Amersham Corp.

Enzymes and reagents

Acrylamide, restriction enzymes and agarose were obtained from Invitrogen. DE52 and P-11 cellulose were purchased from Whatman. Trypsin inhibitor-agarose and single-stranded DNA-cellulose were from Sigma. Sephacryl S-200 was from Pharmacia Biotech Inc. The *B.cereus* ATCC 14579 genomic DNA was purchased from ATCC. Advantage2 polymerase was purchased from Clontech.

Protein determination

Protein concentration was determined by the method of Bradford using a BioRad protein assay kit.

Radiolabeling of oligonucleotides

Oligonucleotides were 5′ end-labeled using bacteriophage T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP

following the manufacturer's recommendations. The labeled oligonucleotides were fractionated on a polyacrylamide gel. The region containing the labeled oligonucleotide was excised, and the DNA was isolated by direct elution of the fragment into 10 mM Tris–HCl (pH 7.5 at 22°C) and 1 mM EDTA. The radiolabeled oligonucleotides were diluted to a specific activity of 5000 c.p.m./pmol by the addition of excess unlabeled oligonucleotide.

Construction of the *bc*TopoIIIα and *bc*Topo I expression vectors

The gene encoding the *bc*Topo IIIα protein was amplified by the PCR using the following primer sequences: 5′-GGAGC-**CATATG**AAATTAATTATTGCCGAG-3′ (N-terminal) and 5′-ATCCCG**CTCGAG**TTAATTCTCAAATACAAAATTAA-TCG-3′ (C-terminal). The amplified product contained a NdeI restriction site (boldface) encompassing the initiation codon of *bc*Topo IIIα gene (*topBα*), and a XhoI site (boldface) after the termination codon of the *topBα* open reading frame (ORF). The amplified product was treated with NdeI and XhoI and ligated with NdeI/XhoI-digested plasmid vector pET21b (pTopo3α). The gene encoding the *bc*Topo I protein was also amplified by the PCR using the following primer sequences: 5′-CCG**TCTAGA**AATAATTTTGTTTAACTT-TAAG*AAGGAGAGG*CTAGC*ATG*TCAGATTACCTCGTA-ATCG*TGG*-3′ (N-terminal) and 5′-ATCCCG**CTCGAG**TCA-CATTTGTTGTTCTTCTTC-3′ (C-terminal). The amplified product contained an XbaI restriction site (boldface) and ribosome binding site (underlined) preceding the initiation codon (italicized) of the *bc*Topo I gene (*topA*), and an XhoI site (boldface) after the termination codon of the *topA* ORF. The amplified product was treated with XbaI and XhoI and ligated with XbaI/XhoI-digested plasmid vector pET21b (pTopo1). The expression vectors were sequenced to ensure that no mutations occurred during the PCR amplification process.

Sequencing errors were found in the published sequence of *bc*Topo I gene. Based on the published sequence, the ORF (3811119–3809131 of complete genome) that encodes *bc*Topo I lacks the first 30 amino acids that are present in other homologs. The region flanking the putative start codon of 3811119 has been amplified by PCR using *Pfu* DNA polymerase. Sequencing of this fragment reveals that an extra C is present at position 38111144 of published sequence. This leads to a +1 frame shift and causes a premature stop within an ORF that starts at position of 3811210 (ORF 3811210–3811031). Without this base, the correct ORF (3811210–3809131) encodes a full length *bc*Topo I that is almost identical to the homolog from *B.anthracis*. In addition, at several other positions, the DNA sequence from the PCR amplified gene was not consistent with the published sequence; however, the derived amino acids from amplified gene at these sites were identical to that of *bc*Topo I homologs from *B.anthracis* and *B.thuringiensis*. Since these ambiguous sequences may be result of sequencing errors, these sites were not altered. These sites are (the published sequences are shown in underline) 11 Pro (CCT)/Arg (CGT), 125 Pro (CCT)/Leu (CTT), 187 Pro (CCT)/Leu (CTT), 319 Thr (ACT)/Ile (ATT), 321 Ala (GCG)/Val (GTC), 322 Tyr (TAT)/Asp (GAT) and 632 Ser (AGT)/Ile (ATT).

Purification of the *bc*Topo IIIα and *bc*Topo I proteins

The induction of the *bc*Topo IIIα polypeptide was initiated by infection of the expression strain, harboring the pTopo3α plasmid DNA, with bacteriophage λCE6. To prevent any contamination of the *bc*Topo IIIα protein with endogenous *ec*Topo III, it was purified from *E.coli* strain BL21 in which the gene encoding *ec*Topo III (*topB*) had been disrupted. The pellets of induced BL21 cells (1L) were resuspended in 15 ml of BugBuster buffer (Novagen) followed by centrifugation (30 min, 40 000 *g*). The pellets were resuspended with 15 ml buffer B (20 mM Tris–HCl, pH 8.0; 1 mM DTT; 1 mM EDTA, pH 8.0; 10% glycerol; 1 M NaCl) followed by centrifugation (30 min, 40 000 *g*). The supernatants containing *bc*Topo IIIα were then dialyzed in 1.5 l buffer A (20 mM Tris–HCl, pH 8.0; 1 mM DTT; 1 mM EDTA, pH 8.0; 10% glycerol) overnight. The desalted crude protein extract was loaded onto 20 ml DE52 cellulose column (2.5 × 10 cm) equilibrated with buffer A. The proteins that bound to DE52 cellulose were step-eluted with 40 ml of buffer A containing 50, 100, 150 and 200 mM NaCl. The 150 and 200 mM NaCl pools were combined and dialyzed against 1.5 l buffer A overnight. The proteins were further purified by single-stranded DNA-cellulose and Sephacryl S-200 chromatography as described previously (29). Proteins bound to single-stranded DNA cellulose were eluted using buffer A containing 1 M NaCl. The same protocol was used to overexpress and purify *bc*Topo I.

Superhelical DNA relaxation assays

Superhelical DNA relaxation reaction mixtures (25 µl) contained 40 mM Tris–HCl buffer (pH 8.0 at 22°C), 1 mM magnesium acetate (pH 7.0), 0.1 mg/ml BSA, 40% (v/v) glycerol, 200 ng of ΦX174 replicative form I DNA (Invitrogen) and the indicated amount of topoisomerase. Reactions were incubated at 52°C for 10 min for *ec*Topo III and *bc*Topo IIIα and 37°C for 30 min for *ec*Topo I and *bc*Topo I. The reaction products were separated on an agarose gel and visualized by staining with ethidium bromide as described previously (28).

oriC DNA replication assay

The replication of *oriC*-containing DNA *in vitro* was performed as described previously (8). The replication products were separated by agarose gel electrophoresis. Replication products were visualized and the percentage of replication products existing as form II (nicked or gapped circular) molecules was quantified using a Fuji BAS 1000 phosphoimager.

Topoisomerase-mediated single-stranded DNA binding assay

Reaction mixtures (10 µl) contained 40 mM Tris–HCl buffer (pH 8.0 at 22°C), 0.1 mg/ml BSA, 1 mM magnesium acetate (pH 7.0) and 5 pmol of a radiolabeled 28-base oligonucleotide. The oligonucleotide was 5′-TTTTCATCCCGAAGTTGCGG-CTCATTCT-3′. The reactions were incubated for 5 min at 37°C, and were stopped by the addition of 10 µl of 20% glycerol and 40 mM EDTA. The reaction products (10 µl) were separated by electrophoresis on a 0.5× TBE polyacrylamide gel (19:1, 12%). The gels were then dried and autoradiographed.

Topoisomerase-induced DNA cleavage assay

Reaction mixtures (10 µl) contained 40 mM Tris–HCl buffer (pH 8.0 at 22°C), 0.1 mg/ml BSA, 1 mM magnesium acetate (pH 7.0) and 5 pmol of a radiolabeled 28-base oligonucleotide. The oligonucleotide was as described above. Reactions containing the indicated amounts of *ec*Topo I, *ec*Topo III, *bc*Topo I and *bc*Topo IIIα were incubated for 3 min at 37°C, and were stopped by the addition of SDS to 2%. The reactions were adjusted to 45% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol, and heat-denatured for 5 min at 90°C. The reaction products were separated by electrophoresis on a 15% 1× TBE polyacrylamide gel (19:1) containing 50% (w/v) urea. The gels were then dried and autoradiographed.

Bacteriophage P1 transduction

Bacteriophage P1 lysates were prepared from *E.coli* strain K38 *topB:kan^r^*. This strain contains a *topB* gene disruption where a kanamycin resistance cassette has been inserted into an EcoRV site within the *topB* gene. *E.coli* strain DM750, harboring the appropriate plasmid DNA, was grown overnight in Luria–Bertani (LB) medium (5 ml) containing 200 µg/ml ampicillin. The cells were harvested and resuspended in 5 ml of sterile 0.1 M magnesium sulfate and 5 mM calcium chloride. The resuspended cells were then incubated for 20 min at 37°C with gentle agitation. The cells (0.1 ml) were mixed with 0.1 ml of the P1 lysate and allowed to stand for 20 min at 37°C. Sodium citrate (0.2 ml of a 1 M solution) was then added to the mixture, and 0.2 ml of the mixture was distributed onto LB agar plates containing 50 µg/ml kanamycin and 100 µg/ml ampicillin. The plates were incubated at 37°C, and the number of kanamycin/ampicillin-resistant colonies was recorded after 18 h. The mixture was also plated on agar medium containing only ampicillin to determine the total number of recipient cells.

RESULTS

B.cereus encodes both *E.coli* Topo I and Topo III homologs

A sequence comparison of *ec*Topo III using BlastP against the *B.cereus* ATCC 14579 complete genomic sequence revealed that this organism potentially contains three chromosomally encoded type IA topoisomerases. Based on a homology comparison and biochemical properties (this study), these proteins have been annotated as topoisomerase I (*bc*Topo I, gi: 30021912), topoisomerase IIIα (*bc*Topo IIIα, gi: 30019974) and topoisomerase IIIβ (*bc*Topo IIIβ, gi: 30018925), respectively. *bc*Topo I is composed of 692 amino acids with a molecular weight of 79.2 kDa and a predicated PI of 8.2 (the *bc*Topo I derived from the published genomic sequence of *B.cereus* ATCC 14579 lacks the first N-terminal 30 amino acids owing to sequencing error as described in Materials and Methods). *bc*Topo I exhibits 36% identity and 50% similarity with *ec*Topo I. Similar to *ec*Topo I (30), it possesses three tetracysteine zinc finger motifs; however, the C-terminal region of *ec*Topo I (amino acid 743–865) has an additional 123 amino acids that are not present in *bc*Topo I. In addition, a fragment that ranges from residue 41 to 69 in *ec*Topo I,

```
bcTopo IIIα  601  VEMSEKWDFTGLHVESIERKGSKFTTGKKVGSCKKCDGDVIDKSTFYGCSNYNTTQCDFTISKKILSKTISQKNMKKLLK

bcTopo IIIα  681  GEKTDLIKGFKKGEKTFDAKLEWKDNKINFVFEN
```

Figure 1. The putative zinc-finger motif of the C-terminal region of *bc*Topo IIIα. The charged residues (24 positively charged residues and 16 negatively charged residues) are in boldface, the conserved cysteine residues that form a putative zinc-finger motif are shadowed in gray.

including eight positively charged residues and three negatively charged residues, is also missing in *bc*Topo I. A further sequence alignment indicates that this region is absent in many *ec*Topo I-like enzymes. Interestingly, this region appears to be disordered in the X-ray crystal structure of *ec*Topo I (31).

*bc*Topo IIIα is composed of 714 amino acids with a molecular weight of 81.5 kDa and a predicated PI of 9.4. Among the first 600 amino acids, *ec*Topo III and *bc*Topo IIIα exhibit 32% identity and 55% similarity. Interestingly, unlike *ec*Topo III, *bc*Topo IIIα possesses a highly charged C-terminal region with a putative Zinc finger motif (Figure 1) that includes 24 positively charged and 16 negatively charged residues among the last 114 C-terminal amino acids. Besides *bc*Topo I and *bc*Topo IIIα, another type IA topoisomerase encoded by the *B.cereus* genome is annotated *bc*Topo IIIβ. Although *bc*Topo IIIβ shares 35% sequence identity and 55% sequence similarity with *ec*Topo III, it is more similar to *Bacillus subtilis* iopoisomerase III (64% sequence identity and 80% sequence similarity) based on sequence alignment (Z. Li and R. DiGate, manuscript submitted).

*bc*Topo I and *bc*Topo IIIα exhibit different biochemical properties

In order to characterize the biochemical activities of *bc*Topo I and *bc*Topo IIIα, the genes encoding these enzymes have been cloned into the T7 RNA polymerase-based expression vector pET21b as described in Materials and Methods. *E.coli* BL21 (*topB*) cells harboring the appropriate expression plasmid were induced by λCE6 phage. The *bc*Topo I and IIIα were purified to apparent homogeneity following a modified topoisomerase purification protocol (Materials and Methods) and their purity was confirmed by SDS–PAGE (Figure 2). Although these proteins are all positively charged at pH 8.0 based on their predicted PI, they bound to DE52 anion exchange cellulose tightly and were eluted only in the presence of 150 mM NaCl. Since both proteins possess efficient single-stranded DNA binding activities, they may bind to DE52 cellulose through their interaction with contaminating nucleic acid or they may also bind to the resin through locally negatively charged regions within the enzyme itself.

The DNA relaxation activity of highly purified *bc*Topo I and *bc*Topo IIIα have been assessed (Figure 3). Although both enzymes exhibit relaxation activity, their mechanism appears to be slightly different. *bc*Topo I relaxes supercoiled DNA efficiently generating a processive pattern of topoisomers (Figure 3A). Fully relaxed DNA species appear even at a low protein concentration (2.2 nM, ~1:1 enzyme to DNA substrate). In addition, it is also able to convert the supercoiled DNA substrate to almost fully relaxed form at higher concentrations. In contrast, *bc*Topo IIIα partially relaxes supercoiled DNA in a much less processive manner (Figure 3B), although it eventually converts supercoiled DNA to topoisomers that are close to fully relaxed. Slightly higher concentrations of
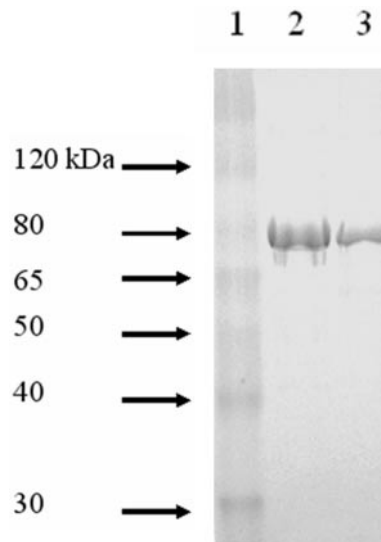

Figure 2. SDS–PAGE of purified *bc*Topo I and *bc*Topo IIIα. Lane 1, protein mass markers; lane 2, *bc*Topo I; lane 3, *bc*Topo IIIα. Purified enzymes (1.0 µg) were electrophoresed through a 10% polyacrylamide gel in the presence of 0.1% SDS. The protein was visualized by Coomassie blue staining.

*bc*Topo I and *bc*Topo IIIα in the reactions result in the decrease of relaxation activity (data not shown). This may be due to the result of protein aggregation.

The pH, Mg^{2+} and temperature characteristics of *bc*Topo I and IIIα have been determined based on a topoisomerase-mediated supercoiled DNA relaxation assay (Figure 4). *bc*Topo I exhibits consistent DNA relaxation activity within a pH range from 6.5 to 10.4 (Figure 4A), whereas, *bc*Topo IIIα-catalyzed DNA relaxation activity remains consistent from a pH ranging from pH 8.0 to 9.8 (Figure 4B). Interestingly, the DNA relaxation activity of *bc*Topo IIIα is slightly inhibited at pH 7.5 and 10.4. Similar to *ec*Topo I (32), high concentrations of Mg^{2+} stimulate the DNA relaxation activity of *bc*Topo I (Figure 4C). Although the enzyme is able to relax DNA at 0.1 mM of Mg^{2+}, it displays a distributive pattern rather than the processive pattern that appears at high concentrations of Mg^{2+}. The optimal concentration of Mg^{2+} is 2.5 mM, higher concentrations of Mg^{2+} (up to 20 mM) slightly inhibit the DNA relaxation activity of *bc*Topo I. Unlike *ec*Topo III, whose DNA relaxation activity is inhibited by high concentrations of Mg^{2+} (>2 mM), *bc*Topo IIIα is capable of relaxing supercoiled DNA at a broad range of Mg^{2+} concentrations (Figure 4D). *bc*Topo IIIα also converts supercoiled DNA molecules into partially relaxed topoisomers that are close to that of the original supercoiled forms without addition of Mg^{2+} (Figure 4D, lane 2). This may be due to a trace amount of Mg^{2+} that remains during the protein purification process since the enzyme completely looses its relaxation activity in the presence of 1 mM EDTA. The optimal Mg^{2+} concentration
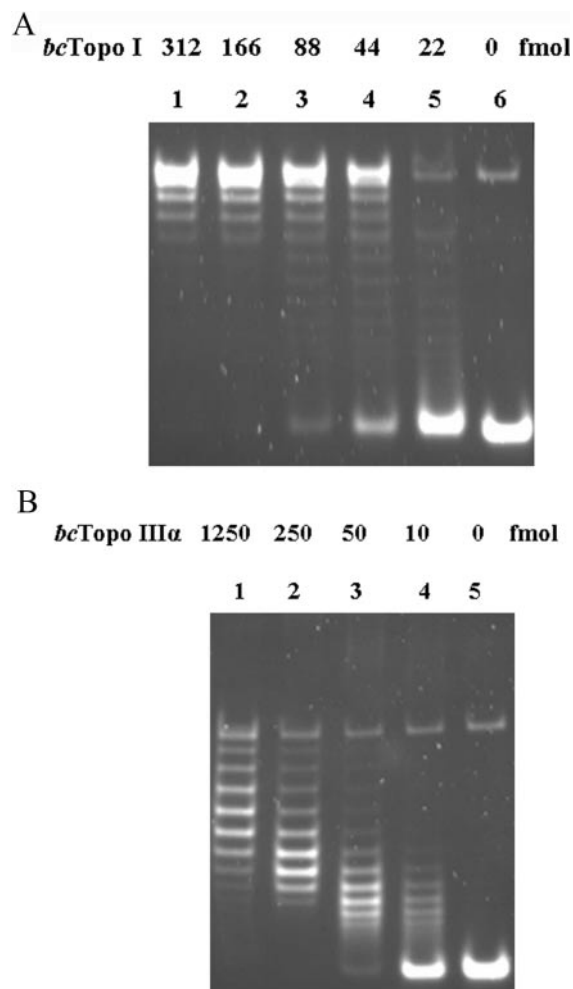
Figure 3. (**A**) DNA relaxation assay of *bc*Topo I. Reactions (25 μl) contained 5 mM Mg^{2+}, 1 mM DTT, 30% glycerol, 100 ng/μl BSA and 40 mM Tris–HCl (pH 8.0 at 22°C) with indicated amount of *bc*Topo I. Lanes 1–6: 312, 166, 88, 44, 22 and 0 fmol of *bc*Topo I, respectively. The reactions were incubated at 37°C for 30 min. The reaction products were eletrophoresed through a 1% 1× TAE agarose gel and the DNA topoisomers were visualized by ethidium bromide staining. (**B**) DNA relaxation assay of bcTopo IIIα. Lanes 1–5: 1.25, 0.25, 0.05, 0.01 and 0 pmol of *bc*Topo IIIα, respectively. The reactions (containing 2.5 mM Mg^{2+} and as described in Materials and Methods) were incubated at 52°C for 10 min and reaction products were separated and visualized as described above.

for *bc*Topo IIIα-catalyzed DNA relaxation is between 1 and 10 mM. In addition, *bc*Topo IIIα is able to tolerate concentrations of Mg^{2+} up to 20 mM with only a slight decrease in relaxation activity. High temperature inhibits the DNA relaxation activity of *bc*Topo I (Figure 4E). In contrast, high temperature is needed for the optimal DNA relaxation activity of *bc*Topo IIIα (Figure 4F). The optimal reaction temperature for *bc*Topo IIIα-catalyzed DNA relaxation is 52°C, the same as that of *ec*Topo III. Temperatures >37°C reduce the activity of *bc*Topo I.

*bc*Topo I and *bc*Topo IIIα possess distinct single-stranded DNA binding activity and exhibit different DNA cleavage site specificity

To catalyze DNA relaxation, type IA topoisomerases need to bind and transiently cleave single-stranded DNA present in superhelical DNA molecules. Topoisomerase-mediated single-stranded DNA mobility shift assays have been applied to analyze the DNA binding activity of *bc*Topo I and *bc*Topo IIIα (Figure 5A). A 28 base ^{32}P-labeled oligonucleotide containing *ec*Topo III cleavage sites was used as the substrate. Although *ec*Topo I and *bc*Topo I possess three Zinc finger motifs, *bc*Topo I does not have an extra C-terminal region present in *ec*Topo I. *ec*Topo I and *bc*Topo I exhibit comparable single-stranded DNA binding activity. Furthermore, both enzymes tend to form a protein–DNA supershift complex at the high concentration of protein. This suggests that last 123 residues of *ec*Topo I C-terminal region may not be critical for single-stranded DNA binding.

*ec*Topo III and *bc*Topo IIIα possess distinct C-terminal regions and they exhibit different single–stranded DNA binding activities. Having a putative zinc finger motif and a highly charged C-terminal region, *bc*Topo IIIα bound to single-stranded DNA is capable of forming a unique supershift complex. The composition of this supershift complex is not clear, multiple proteins may bind to a single DNA substrate coordinately or individually, or a single protein may bind to multiple oligonucleotides simultaneously.

The same radiolabeled 28 base oligonucleotides used in DNA binding assay has also been used as a substrate to assess the cleavage site specificity of *bc*Topo I, *bc*Topo IIIα, *ec*Topo I and *ec*Topo III (Figure 5B). *bc*Topo I and *ec*Topo I share three common cleavage sites but the enzymes prefer different subsets of the cleavage sites. *bc*Topo IIIα and *ec*Topo III also exhibit common cleavage sites with different preferences. *bc*Topo I and *bc*Topo IIIα do not share the same major cleavage sites using this substrate. Besides supercoiled DNA relaxation efficiency, the single-stranded DNA cleavage specificity may also be considered as another property that distinguishes *ec*Topo I-like topoisomerase from *ec*Topo III-like enzymes.

Based on the X-ray crystal structure of *ec*Topo III (33), residues from 165 to 195 should fold into a helix–turn–helix structure and are part of the DNA binding cavity. These residues may contribute to protein–DNA interactions. Further sequence comparison of this fragment with other *ec*Topo III-like enzymes reveals that residues of this region are generally conserved (Figure 6). Interestingly, the corresponding regions in *ec*Topo I-like enzymes are virtually identical. Furthermore, several conserved residues present in *ec*Topo III-like proteins are different from those present in *ec*Topo I-like proteins. Therefore, this region may not only be critical for DNA binding for type IA topoisomerases, but may serve to delineate Topo-I like enzymes.

*bc*Topo IIIα is a potent DNA decatenase, but not *bc*Topo I

*ec*Topo III is a potent decatenase that is capable of separating multiple interlinked circular DNA dimers. A unique loop, located near the large central cavity present in the enzyme, has been shown to play an essential role in *ec*Topo III-catalyzed DNA decatenation and termed as 'decatenation loop' (9). Many *ec*Topo III-like enzymes also possess the decatenation loop and exhibit DNA decatenation activity. *bc*Topo IIIα also possesses the decatenation loop and it shares similar biochemical properties with *ec*Topo III. *bc*Topo IIIα
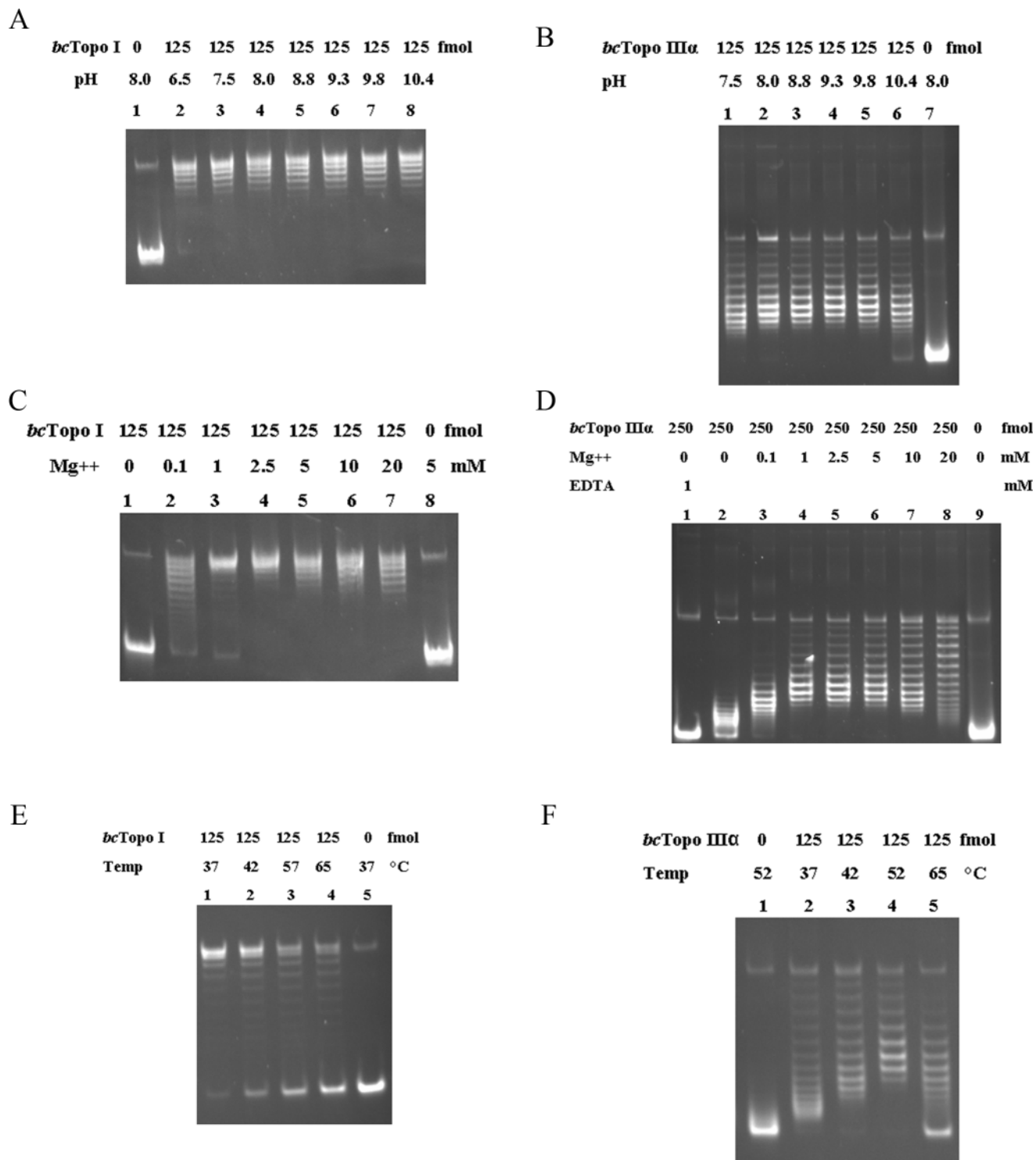
Figure 4. (**A**) Effect of pH on *bc*Topo I-mediated DNA relaxation. Reactions (25 μl) contained 125 fmol of *bc*Topo I, 2.5 mM Mg^{2+} and 40 mM Tris–HCl with the indicated pH value. Lane 1, no protein; lanes 2–8: Tris–HCl, pH of 6.5, 7.5, 8.0, 8.8, 9.3, 9.8 and 10.4, respectively. (**B**) Effect of pH on bcTopo IIIα-mediated DNA relaxation. Reactions (25 μl) contained 125 fmol of *bc*Topo IIIα, 2.5 mM Mg^{2+} and 40 mM Tris–HCl with the indicated pH value. Lane 7, no protein; lanes 1–6: Tris–HCl, pH of 7.5, 8.0, 8.8, 9.3, 9.8 and 10.4, respectively. (**C**) Effect of Mg^{2+} concentration on *bc*Topo I-mediated DNA relaxation. Reactions were performed as described above except with different Mg^{2+} concentrations. Lanes 1–7: 0, 0.1, 1, 2.5, 5, 10 and 20 mM Mg^{2+}, respectively. Lane 8, no protein. (**D**) Effect of Mg^{2+} on bcTopo IIIα-mediated DNA relaxation. Lane 1, 1 mM of EDTA without Mg^{2+}; lanes 2–8, 0, 0.1, 1, 2.5, 5, 10 and 20 mM Mg^{2+}, respectively; Lane 9, no protein. Reactions were performed as described above except with different Mg^{2+} concentrations. (**E**) Effect of temperature on *bc*Topo I-mediated DNA relaxation. Lanes 1–4, reactions were performed at 37, 42, 52 and 65°C, respectively; lane 5, no protein. Reactions were performed as described above except for different incubation temperatures. (**F**) Effect of temperature on *bc*Topo IIIα-mediated DNA relaxation. Lane 1, no protein; lanes 2–5, reactions were performed at 37, 42, 52 and 65°C, respectively. Reactions were performed as described above except for different incubation temperatures.
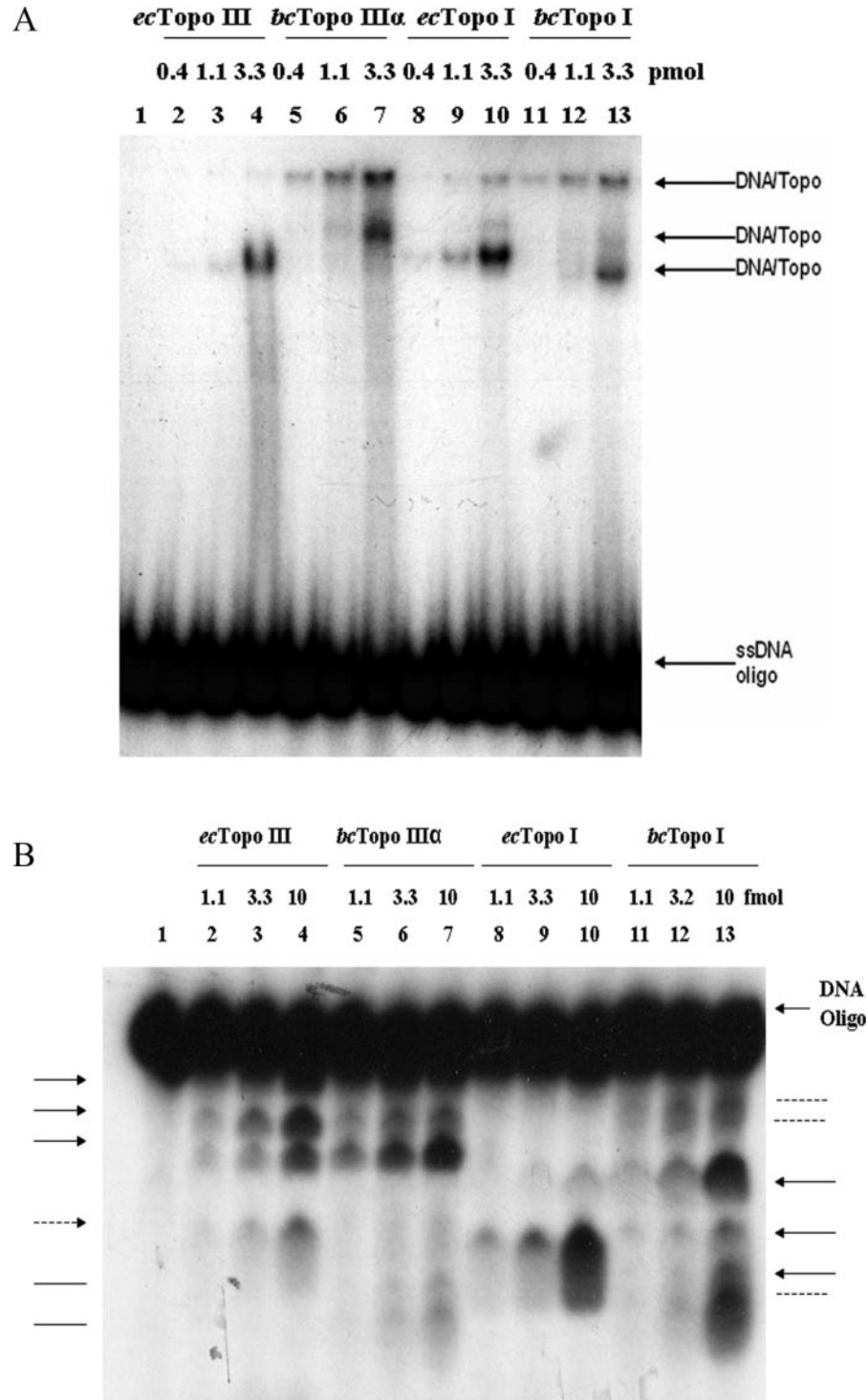
Figure 5. (**A**) Comparison of the single-stranded DNA binding activities of *bc*Topo IIIβ, *ec*Topo III, *bc*Topo I and *ec*Topo I. The assay was performed as described in Materials and Methods. Reactions (10 μl) contained 5 pmol of ^{32}P-radiolabeled oligonucleotide, 1 mM Mg^{2+}, 1 mM DTT, 30% glycerol, 100 ng/μl BSA and 40 mM Tris–HCl (pH 8.0) and the indicated amount of protein. Lane 1, no protein; lanes 2–4: 0.4, 1.1 and 3.3 pmol of *ec*Topo III; lanes 5–7: 0.4, 1.1 and 3.3 pmol of *bc*Topo IIIα; lanes 8–10: 0.4, 1.1 and 3.3 pmol of *ec*Topo I; lanes 11–13, 0.4, 1.1 and 3.3 pmol of *bc*Topo I. The reactions were eletrophoresed through 12% polyacrylamide gel in 0.5× TBE and visualized by autoradiography. (**B**) Comparison of the single-stranded DNA cleavage site specificities of *bc*Topo IIIβ, *ec*Topo III, *bc*Topo I and *ec*Topo I. The assay was performed as described in Materials and Methods. Reactions (10 μl) contained 5 pmol of ^{32}P radiolabeled oligonucleotide, 1 mM Mg^{2+}, 1 mM DTT, 30% glycerol, 100 ng/μl BSA and 40 mM Tris–HCl (pH 8.0 at 22°C) and indicated amount of protein. Lane 1, no protein; lanes 2–4: 1.1, 3.3 and 10 pmol of *ec*Topo III, respectively; lanes 5–7: 1.1, 3.3 and 10 pmol of *bc*Topo IIIα, respectively; lanes 8–10: 1.1, 3.3 and 10 pmol of *ec*Topo I, respectively; lanes 11–13: 1.1, 3.3 and 10 pmol of *bc*Topo I, respectively. The reactions were electrophoresed through a 15% polyacrylamide gel in 1× TBE with 50% urea and visualized by autoradiography. The common cleavage sites of *bc*Topo IIIα and *ec*Topo III are indicated by right arrow; The common cleavage sites of *bc*Topo I and *ec*Topo I are indicated by left arrow; unique cleavage sites created by *bc*Topo IIIα are indicated by dash; unique cleavage sites created by *ec*Topo III are indicated by dotted arrow; unique cleavage sites created by *bc*Topo I are indicated by dots.

```
E. coli          Topo III    162  ALARARADWLYGINMTRAYTILGRNAGYQG-VLSVGRVQTP 201
B. cereus        Topo IIIα   164  AYTRSCADWVVGMNASRVFSILLKKKGMND-VFSAGRVQTP 203
S. epidermidis   Topo IIIα   171  AEARSIADWLVGMNFTRYVTLEMKQLGIDEGVFSVGRVQTP 209
pRp4             TraE        159  ALARSVADAIYGLSMTRAYTIPAKTKGYKG-VLSVGRVQTP 198
pXO2-76/77       Topo        165  AQARQISDWAIGMNASRLYTLLLRQKGVQG-VYSVGRVQTP 204
pXO1-142         Topo        167  AKARQYLDYLLGMNITRGCTTKLAQNKF---LLSSGRVQMC 204
E. faecalis      Topo IIIα   161  ALARAKADWLVGLNVTRALTV-----KYQD-NLSAGRVQTP 195
E. faecalis      Topo IIIβ   158  ALCRAKADWLIGINGTRLFTT-----LYQGKSLNVGRVQTP 193
S. aureus        Topo III    159  ALARSEADWIVGINATRALTT-----KYDA-QLSLGRVQTP 193
S. epidermidis   Topo IIIβ   159  ALARSEADWIVGINATRALTT-----KYDA-QLSLGRVQTP 193
B. subtilis      Topo III    160  AVARAEADWIVGINATRALTT-----KFNA-QLSCGRVQTP 194
B. cereus        Topo IIIβ   160  AVARSEADWYIGLNATRALTT-----RFNA-QLNCGRVQTP 194
                                  X  +    +X  +X  +X  X            + +++++X
E. coil          Topo I      164  AQQARRFMDRVVGYMVSPLLW------KKIARGLSAGRVQSV 199
B. cereus        Topo I      135  AQQARRILDRLVGYNISPLLW------KKVKKGLSAGRVQSV 170
B. subtilis      Topo I      135  AQQARRILDRLVGYKISPILW------KKVKKGLSAGRVQSV 170
S. aureus        Topo I      135  AQQARRILDRLVGYNISPVLW------KKVKKGLSAGRVQSV 170
S. epidermidis   Topo I      135  AQQARRILDRLVGYNISPVLW------KKVKKGLSAGRVQSV 170
E. faecalis      Topo I      136  AQQARRILDRLVGYSISPILW------RKVKKGLSAGRVQSV 171
```

Figure 6. Sequence alignment of a fragment of DNA binding region conserved in type IA topoisomerases. Protein sequence comparisons were performed using the BlastP program (19). The same conserved residues present in both *ec*Topo I-like and *ec*Topo III-like proteins are indicated by plus; the residues that are conserved in *ec*Topo I-like and *ec*Topo III-like proteins but are different between two groups are indicated by cross.

is able to resolve *oriC* based plasmid DNA replication intermediates *in vitro* (Figure 7). Although *bc*Topo I is very efficient at the relaxation negatively supercoiled DNA, like *ec*Topo I, it is not an efficient decatenase. This implies that different catalytic mechanisms have been applied by *bc*Topo I and *bc*Topo IIIα to perform intramolecular (which results in relaxation) and intermolecular (which results in decatenation) strand passage.

*bc*Topo I and *bc*Topo IIIα are able to substitute for the loss of *ec*Topo III *in vivo*

It has been proposed that *ec*Topo I in conjugation with DNA gyrase regulates superhelical density in *E.coli*. Therefore, cells with a deletion of *topA* (the gene encoding *ec*Topo I) can only be isolated in the presence of a defective gyrase activity or other compensatory mutations (6,7). Furthermore, it has been shown that introducing a *topB* deletion to DM750 cells (a *topA* deletion strain) is difficult unless the cell harbors a *topB* expression plasmid (28). Under these circumstances the transcription and translation of the plasmid encoded *topB* gene presumably provides enough *ec*Topo III protein for cells to survive.

If *bc*Topo I and *bc*Topo IIIα can perform similar functions as their *E.coli* counterparts *in vivo*, *bc*Topo IIIα should be able to substitute for the loss of *ec*Topo III. To confirm this assumption, P1 phage transduction has been used to introduce a *topB* disruption into the DM750 chromosome. For this study the genes encoding *bc*Topo I (pTopo1) and *bc*Topo IIIα (pTopo3α) were subcloned into plasmid pET3c (resulting in pBT1 and pBT3α, respectively). DM750 cells harboring plasmids pDE1 (containing the *E.coli topB* gene in pET3c), pTI1 (containing the *E.coli topA* gene in pET3c), pBT1 and pBT3α were transduced to *topB:kan^r* by P1 phage. Consistent with previous observations (28), cells containing pTI1 can not

be transduced to *topB:kan^r*; however, cells harboring either pDE1, pBT3α and pBT1 can be transduced to *topB:kan^r* (Table 1). The transduction efficiency of pBT3α containing cells is increased ∼2-fold compared with that of pDE1 containing cells, whereas the transduction efficiency of pBT1 containing cells is 3-fold reduced compared with that of pDE1 containing cells.

DISCUSSION

B.anthracis, *B.cereus* and *B.thuringiensis* are closely related. These organisms all have three chromosomally encoded type IA topoisomerases. Moreover, bacteria such as *C.pertringens*, *C.tetani*, *E.faecalis* and *S.epidermidis* also contain three chromosome copies of type IA topoisomerases that exhibit extensive amino acid sequence similarity with *bc*Topo I, *bc*Topo IIIα and *bc*Topo IIIβ, respectively (Table 2). In addition, pXO1 and pXO2 plasmids, harbored by *B.anthracis*, may encode two additional type IA topoisomerases. It has been reported that pXO1 gene 142 encodes a type IA topoisomerase termed TopX (34). Based on the published sequence, the polypeptides derived from the pXO2 gene 77 and 76 show sequence similarity with N- and C-terminal regions of a type IA topoisomerase, respectively (35). If *amber* suppression were to occur (an *amber* stop codon is present at the end of gene 77), the ORF beginning at the gene 77 start codon would continue through to the gene 76 stop codon and would encode a complete type IA topoisomerase. Interestingly, this putative topoisomerase shows 46% identity and 64% similarity with hypothetical protein γ of plasmid *Streptococcus pyogenes* plasmid pDB101 (a type IA topoisomerase) (36) and also exhibits 59% sequence similarity with *bc*Topo IIIα. It remains to be determined whether the stop codon of gene 77 actually exits (i.e. there may be a sequencing error) or whether
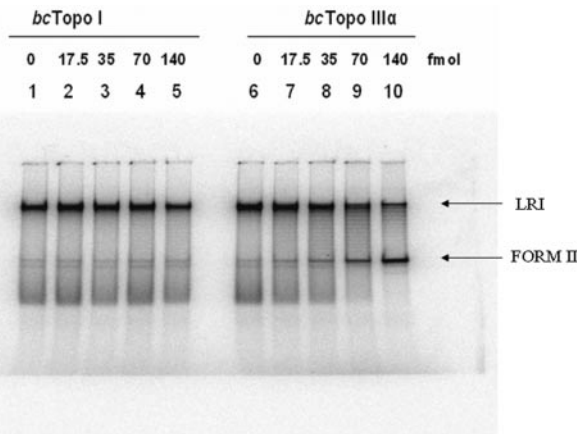
Figure 7. Resolution of the *oriC* replication intermediates by *bc*Topo I and *bc*Topo IIIα. The reactions (12.5 µl) contained no protein (lanes 1 and 6); 17.5 fmol of *bc*Topo I or *bc*Topo IIIα (lanes 2 and 6); 35 fmol of *bc*Topo I or *bc*Topo IIIα (lanes 3 and 7); 70 fmol of *bc*Topo I or *bc*Topo IIIα (lanes 4 and 8); and 140 fmol of *bc*Topo I or *bc*Topo IIIα (lanes 5 and 10). The fully decatenated form II molecules and late replication intermediate (LRI) are indicated.

Table 1. P1 transduction frequencies of *E.coli* strain DM750 harboring different plasmids

18 h incubation at 37°C	Plasmid				
	None	pDE1	pTI1	pBT3α	pBT1
Transduction frequency ($\times 10^{-7}$)	N/A	3.61	N/A	6.34	1.22

The data represents the average transduction frequency of three experiments. The total numbers of transductants in three experiments were 477 (for strains contain pDE1), 873 (for strain contain pBT3α) and 140 (for strain contain pBT1). The *topB:kan*[r] disruptions were confirmed by PCR amplification of seven independent transductants from each group.

Table 2. Many Gram-positive bacteria possess multiple type IA topoisomerases

Organism	Gene	Size amino acid	Bits score	References
E.coli K12	*topB*	653	1238	(5)
E.coli K12	*topA*	865	122	(4)
B.anthracis	*topBα*	714	340	(20)
B.anthracis	*topBβ*	729	320	(20)
B.anthracis	*pXO2-77/76*[a]	676	289	(34)
B.anthracis	*pXO1-142*[b]	887	199	(32)
B.anthracis	*topA*	692	105	(20)
B.cereus	*topBα*	714	337	(21)
B.cereus	*topBβ*	729	328	(21)
B.cereus	*topA*	692	107	(21)
C.tetani	*topBα*	721	228	(24)
C.tetani	*topBβ*	730	323	(24)
C.tetani	*topA*	696	115	(24)
E.faecalis	*topBα*	705	315	(25)
E.faecalis	*topBβ*	693	262	(25)
E.faecalis	*topA*	692	99	(25)
S.epidermidis	*topBα*	718	249	(26)
S.epidermidis	*topBβ*	711	272	(26)
S.epidermidis	*topA*	689	129	(26)

Amino acids sequence comparison of *E.coli* Topo III (*topB*) in NCBI microbial genomic data base were done using BlastP program (19).
[a]A putative topoisomerase encoded by pXO2 plasmid was formed by changing the *amber* stop codon TAG of gene 77 to GAG.
[b]TopX encoded by pXO1 gene 142.

translation can be accomplished through *amber* suppression. In either case, some *Bacillus* species may have up to five type IA topoisomerases. Other pXO2-like plasmids, such as pAMβ1, found in Gram-positive bacteria also possess an active type IA TOP3. Clearly, it is important to understand the redundancy and different functions of these enzymes.

Two of the *B.cereus* type IA topoisomerases have been purified and characterized in this work. *bc*Topo I shares extensive sequence similarity with *ec*Topo I (including three C-terminal zinc finger motifs); however, its C-terminal region is shorter (it lacks the last 123 amino acids at C-terminus of *ec*Topo I) and it also lacks a highly positively charged N-terminal loop (residues 41–69) present in *ec*Topo I. Since *bc*Topo I and *ec*Topo I exhibit similar biochemical properties, it appears that these regions may not be critical for the catalytic activity of *bc*Topo I but may relate to the specific biological function of *ec*Topo I *in vivo* (e.g. by mediating protein–protein interactions). *bc*Topo I is efficient in relaxing negatively supercoiled DNA in a processive manner. The DNA relaxation activity of *bc*Topo I, similar to *ec*Topo I, is stimulated by high concentration of Mg^{2+} and is inhibited by high temperature. In addition, *bc*Topo I and *ec*Topo I display comparable single-stranded DNA binding activities and share common DNA cleavage sites. Similar to *ec*Topo I, *bc*Topo I is not capable of resolving multiple interlinked DNA dimers.

*bc*Topo IIIα and *ec*Topo III exhibit extensive protein sequence homology within the first 600 amino acids; however, there is a little homology within their C-terminal sequence. *bc*Topo IIIα has a highly charged C-terminal region including a putative zinc finger motif. This may be involved in protein–protein interactions to form hetero or homo protein complexes. This would support the observation that *bc*Topo IIIα tends to form a unique supershift complex with single-stranded oligonucleotides when compared with *ec*Topo III. In addition, *bc*Topo IIIα, unlike *ec*Topo III, is able to relax supercoiled DNA under a broad range of Mg^{2+} concentrations ranging from a trace amount to 20 mM. Overall, however, *bc*Topo IIIα is clearly an *ec*Topo III-like enzyme. Similar to *ec*Topo III, it is inefficient at the relaxation of negatively supercoiled DNA and the activity is stimulated by high temperature. *bc*Topo IIIα and *ec*Topo III also exhibit similar single-stranded DNA cleavage specificity and more importantly, *bc*Topo IIIα is a potent decatenase, a characteristic that is typical of *ec*Topo III-like enzyme.

According to amino acid sequence alignment of a protein fragment that makes up a part of the DNA binding cavity (*ec*Topo I, amino acid 164–199; *ec*Topo III, amino acid 162–201), *ec*Topo I-like enzymes have an almost identical sequence when compared with *ec*Topo III-like enzymes (that have relatively diverse sequences with conserved residues). This region may serve to distinguish *ec*Topo I-like enzymes from other prokaryotic type IA topoisomerases.

Although *bc*Topo I and *bc*Topo IIIα exhibit distinctly different biochemical properties, they are both able to substitute for the loss of *ec*Topo III in *E.coil* DM750 cells. This is distinct contrast to the results with *ec*Topo I. The inability of *ec*Topo I to compensate for the loss of *ec*Topo III has been observed previously (28). It is possible that this is a result of a perturbation of chromosomal superhelical density owing to the presence of a gyrase compensatory mutation in DM750. This mutation lowers gyrase activity in order to compensate for

the loss of *ec*Topo I activity in the cell (6,7). Introduction of the wild-type gene encoding *ec*Topo I into this strain could lead to extensive relaxation of the host chromosome. We believe that this explanation is unlikely, however. The specific activity of *bc*Topo I and *ec*Topo I is very similar over a broad range of pH and salt concentration (data not shown). It is quite probable that both *bc*Topo I and *ec*Topo I would have the same effect on overall intracellular supercoiling. The basis for the observation may be rooted in protein–protein interactions and the involvement of *ec*Topo I in a process other than the maintenance of superhelical density (37).

Interestingly, similar to the results with *ec*TopoI, *bc*Topo IIIβ is also unable to compensate for the loss of Topo III in DM750 (Z. Li and R. DiGate, manuscript submitted). This indicates the inability of *ec*Topo I is not strictly unique to this enzyme but has its basis in a true functional deficiency. *bc*Topo IIIβ is a unique topoisomerase that is different from *ec*Topo I and III-like enzymes in that it only partially relaxes supercoiled DNA and is not capable of resolving replication intermediates during *oriC* DNA replication *in vitro*. Interestingly, this enzyme is more closely related to topoisomerase III encoded by bacteria such as *B.subtilis* (38), *Bacillus licheniformis* (39), *Listeria monocytogenes* (40), *Oceanobacillus iheyensis* (41) and *Staphylococcus aureus* (42), and topoisomerase IIIβ encoded by *C.pertringens*, *Enterococcus facecalis* and *S.epidermidis* (these organisms encode two Topo III-like proteins).

Prokaryotic type IA topoisomerases may have more diverse functions than simply the maintenance of genomic stability (as has been shown by eukaryotic type IA topoisomerases and *ec*Topo III). These enzymes may have roles in the horizontal gene transfer of promiscuous plasmids or conjugational transposons. Type IA topoisomerases may also function in the process of conjugational DNA transfer, transposon integration (43), plasmid maintenance and plasmid segregation. To deal with stringent environmental stressors such as UV, chemical and free radical damage, bacteria may not only need sophisticated DNA damage repair systems, but also efficient DNA recombination systems to create, adopt and spread endogenous or exogenous mutations. Therefore, different type IA topoisomerases may be required in a variety of DNA repair and recombination processes. In addition, the characteristic cellular development stages such as sporulation and germination of spore-forming Gram-positive bacteria may require DNA replication and recombination systems that are different from those of *E.coli*. It is possible that type IA topoisomerases in concert with different partners fulfill *in vivo* functions that have not yet been identified in *E.coli*.

ACKNOWLEDGEMENTS

Conflict of interest statement. None declared.

REFERENCES

1. Wang,J.C. (1996) DNA topoisomerases. *Annu. Rev. Biochem.*, **65**, 635–692.
2. Champoux,J.J. (2001) DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.*, **70**, 369–413.
3. Wang,J.C. (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nature Rev. Mol. Cell. Biol.*, **3**, 430–440.
4. Tse-Dinh,Y.C. and Wang,J.C. (1986) Complete nucleotide sequence of the topA gene encoding *Escherichia coli* DNA topoisomerase I. *J. Mol. Biol.*, **191**, 321–331.
5. DiGate,R.J. and Marians,K.J. (1989) Molecular cloning and DNA sequence analysis of *Escherichia coli* topB, the gene encoding topoisomerase III. *J. Biol. Chem.*, **264**, 17924–17930.
6. Pruss,G.J., Manes,S.H. and Drlica,K. (1982) *Escherichia coli* DNA topoisomerase I mutants: increased supercoiling is corrected by mutations near gyrase genes. *Cell*, **31**, 35–42.
7. DiNardo,S., Voelkel,K.A., Sternglanz,R., Reynolds,A.E. and Wright,A. (1982) *Escherichia coli* DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. *Cell*, **31**, 43–51.
8. Hiasa,H. and Marians,K.J. (1994) Topoisomerase III, but not topoisomerase I, can support nascent chain elongation during theta-type DNA replication. *J. Biol. Chem.*, **269**, 32655–32659.
9. Li,Z., Mondragon,A., Hiasa,H., Marians,K.J. and DiGate,R.J. (2000) Identification of a unique domain essential for *Escherichia coli* DNA topoisomerase III-catalysed decatenation of replication intermediates. *Mol. Microbiol.*, **35**, 888–895.
10. Whoriskey,S.K., Schofield,M.A. and Miller,J.H. (1991) Isolation and characterization of *Escherichia coli* mutants with altered rates of deletion formation. *Genetics*, **127**, 21–30.
11. Wilson,T.M., Chen,A.D. and Hsieh,T. (2000) Cloning and characterization of Drosophila topoisomerase IIIbeta. Relaxation of hypernegatively supercoiled DNA. *J. Biol. Chem.*, **275**, 1533–1540.
12. Hanai,R., Caron,P.R. and Wang,J.C. (1996) Human TOP3: a single-copy gene encoding DNA topoisomerase III. *Proc. Natl Acad. Sci. USA*, **93**, 3653–3657.
13. Seki,T., Seki,M., Onodera,R., Katada,T. and Enomoto,T. (1998) Cloning of cDNA encoding a novel mouse DNA topoisomerase III (Topo IIIbeta) possessing negatively supercoiled DNA relaxing activity, whose message is highly expressed in the testis. *J. Biol. Chem.*, **273**, 28553–28556.
14. Li,W. and Wang,J.C. (1998) Mammalian DNA topoisomerase IIIalpha is essential in early embryogenesis. *Proc. Natl Acad. Sci. USA*, **95**, 1010–1013.
15. Kwan,K.Y. and Wang,J.C. (2001) Mice lacking DNA topoisomerase IIIbeta develop to maturity but show a reduced mean lifespan. *Proc. Natl Acad. Sci. USA*, **98**, 5717–5721.
16. Kwan,K.Y., Moens,P.B. and Wang,J.C. (2003) Infertility and aneuploidy in mice lacking a type IA DNA topoisomerase III beta. *Proc. Natl Acad. Sci. USA*, **100**, 2526–2531.
17. Wallis,J.W., Chrebet,G., Brodsky,G., Rolfe,M. and Rothstein,R. (1989) A hyper-recombination mutation in *S.cerevisiae* identifies a novel eukaryotic topoisomerase. *Cell*, **58**, 409–419.
18. Maftahi,M., Han,C.S., Langston,L.D., Hope,J.C., Zigouras,N. and Freyer,G.A. (1999) The top3(+) gene is essential in *Schizosaccharomyces pombe* and the lethality associated with its loss is caused by Rad12 helicase activity. *Nucleic Acids Res.*, **27**, 4715–4724.
19. Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.
20. Read,T.D., Peterson,S.N., Tourasse,N., Baillie,L.W., Paulsen,I.T., Nelson,K.E., Tettelin,H., Fouts,D.E., Eisen,J.A., Gill,S.R. *et al.* (2003) The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature*, **423**, 81–86.
21. Ivanova,N., Sorokin,A., Anderson,I., Galleron,N., Candelon,B., Kapatral,V., Bhattacharyya,A., Reznik,G., Mikhailova,N., Lapidus,A. *et al.* (2003) Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature*, **423**, 87–91.
22. Bhatnagar,R. and Batra,S. (2001) Anthrax toxin. *Crit. Rev. Microbiol.*, **27**, 167–200.
23. Hoffmaster,A.R., Ravel,J., Rasko,D.A., Chapman,G.D., Chute,M.D., Marston,C.K., De,B.K., Sacchi,C.T., Fitzgerald,C., Mayer,L.W. *et al.* (2004) Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. *Proc. Natl Acad. Sci. USA*, **101**, 8449–8454.

24. Shimizu,T., Ohtani,K., Hirakawa,H., Ohshima,K., Yamashita,A., Shiba,T., Ogasawara,N., Hattori,M., Kuhara,S. and Hayashi,H. (2002) Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proc. Natl Acad. Sci. USA*, **99**, 996–1001.

25. Bruggemann,H., Baumer,S., Fricke,W.F., Wiezer,A., Liesegang,H., Decker,I., Herzberg,C., Martinez-Arias,R., Merkl,R., Henne,A. *et al.* (2003) The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *Proc. Natl Acad. Sci. USA*, **100**, 1316–1321.

26. Paulsen,I.T., Banerjei,L., Myers,G.S., Nelson,K.E., Seshadri,R., Read,T.D., Fouts,D.E., Eisen,J.A., Gill,S.R., Heidelberg,J.F. *et al.* (2003) Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science*, **299**, 2071–2074.

27. Zhang,Y.Q., Ren,S.X., Li,H.L., Wang,Y.X., Fu,G., Yang,J., Qin,Z.Q., Miao,Y.G., Wang,W.Y., Chen,R.S. *et al.* (2003) Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol. Microbiol.*, **49**, 1577–1593.

28. Li,Z., Hiasa,H., Kumar,U. and DiGate,R.J. (1997) The traE gene of plasmid RP4 encodes a homologue of *Escherichia coli* DNA topoisomerase III. *J. Biol. Chem.*, **272**, 19582–19587.

29. DiGate,R.J. (1999) Overexpression and purification of *Escherichia coli* DNA topoisomerase III. *Methods Mol. Biol.*, **94**, 153–162.

30. Yu,L., Zhu,C.X., Tse-Dinh,Y.C. and Fesik,S.W. (1995) Solution structure of the C-terminal single-stranded DNA-binding domain of *Escherichia coli* topoisomerase I. *Biochemistry*, **34**, 7622–7628.

31. Lima,C.D., Wang,J.C. and Mondragon,A. (1993) Crystallization of a 67 kDa fragment of *Escherichia coli* DNA topoisomerase I. *J. Mol. Biol.*, **232**, 1213–1216.

32. Domanico,P.L. and Tse-Dinh,Y.C. (1991) Mechanistic studies on *E. coli* DNA topoisomerase I: divalent ion effects. *J. Inorg. Biochem.*, **42**, 87–96.

33. Mondragon,A. and DiGate,R. (1999) The structure of *Escherichia coli* DNA topoisomerase III. *Structure Fold Des.*, **7**, 1373–1383.

34. Fouet,A., Sirard,J.C. and Mock,M. (1994) *Bacillus anthracis* pXO1 virulence plasmid encodes a type 1 DNA topoisomerase. *Mol. Microbiol.*, **11**, 471–479.

35. Okinaka,R., Cloud,K., Hampton,O., Hoffmaster,A., Hill,K., Keim,P., Koehler,T., Lamke,G., Kumano,S., Manter,D. *et al.* (1999) Sequence, assembly and analysis of pX01 and pX02. *J. Appl. Microbiol.*, **87**, 261–262.

36. Ceglowski,P., Boitsov,A., Chai,S. and Alonso,J.C. (1993) Analysis of the stabilization system of pSM19035-derived plasmid pBT233 in *Bacillus subtilis*. *Gene*, **136**, 1–12.

37. Phoenix,P., Raymond,M.A., Masse,E. and Drolet,M. (1997) Roles of DNA topoisomerase in the regulation of R-loop formation *in vitro*. *J. Biol. Chem.*, **272**, 1473–1479.

38. Kunst,F., Ogasawara,N., Moszer,I., Albertini,A.M., Alloni,G., Azevedo,V., Bertero,M.G., Bessieres,P., Bolotin,A., Borchert,S. *et al.* (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature*, **390**, 249–256.

39. Veith,B., Herzberg,C., Steckel,S., Feesche,J., Maurer,K.H., Ehrenreich,P., Baumer,S., Henne,A., Liesegang,H., Merkl,R. *et al.* (2004) The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *J. Mol. Microbiol. Biotechnol.*, **7**, 204–211.

40. Nelson,K.E., Fouts,D.E., Mongodin,E.F., Ravel,J., DeBoy,R.T., Kolonay,J.F., Rasko,D.A., Angiuoli,S.V., Gill,S.R., Paulsen,I.T. *et al.* (2004) Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Res.*, **32**, 2386–2395.

41. Takami,H., Takaki,Y. and Uchiyama,I. (2002) Genome sequence of *Oceanobacillus iheyensis* isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments. *Nucleic Acids Res.*, **30**, 3927–3935.

42. Baba,T., Takeuchi,F., Kuroda,M., Yuzawa,H., Aoki,K., Oguchi,A., Nagai,Y., Iwama,N., Asano,K., Naimi,T. *et al.* (2002) Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet*, **359**, 1819–1827.

43. Sutanto,Y., Shoemaker,N.B., Gardner,J.F. and Salyers,A.A. (2002) Characterization of Exc, a novel protein required for the excision of Bacteroides conjugative transposon. *Mol. Microbiol.*, **46**, 1239–1246.