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

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**ABSTRACT**

The *Bacillus cereus* genome possesses three type IA topoisomerase genes. These genes, encoding DNA topoisomerase I and IIIα (bcTopo I, bcTopo 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 (ecTopo I, ecTopo III). bcTopo I is capable of efficiently relaxing negatively supercoiled DNA in the presence of Mg2+ but does not possess an efficient DNA decatenation activity. bcTopo IIIα is an active topoisomerase that is capable of relaxing supercoiled DNA at a broad range of Mg2+ concentrations; however, its DNA relaxation activity is not as efficient as that of bcTopo I. In addition, bcTopo III is a potent DNA decatenase that resolves oriC-based plasmid replication intermediates in vitro. Interestingly, bcTopo I and bcTopo IIIα are both able to compensate for the loss of ecTopo III in *E.coli* cells that lack ecTopo I. In contrast, ecTopo I cannot substitute for ecTopo 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, ecTopo I (4) and ecTopo III (5). Among all characterized type IA topoisomerases, ecTopo I-like enzymes are capable of relaxing negatively supercoiled DNA efficiently. Therefore, ecTopo I in conjunction with DNA gyrase (a type IIA enzyme) functions in the regulation of chromosomal superhelicity (6,7). Although ecTopo III relays 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 ecTopo 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 TOP3β 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 ecTopo 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 Clostridium perfringens (24), Clostridium 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 ecTopo I and ecTopo 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 ecTopo IIIα 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. Tryptsin 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-32P]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 bcTopoIIIα and bcTopo I expression vectors

The gene encoding the bcTopo IIIα protein was amplified by the PCR using the following primer sequences: 5′-GGAGCCATATGAAAAATATTATGCGAG-3′ (N-terminal) and 5′-ATCCCGCTCGAGTTAATTCTCTAAATAAATTAA-TCG-3′ (C-terminal). The amplified product contained a NdeI restriction site (boldface) encompassing the initiation codon of bcTopo IIIα gene (stop branch), 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 bcTopo I protein was also amplified by the PCR using the following primer sequences: 5′-CCGTCTAGAATAATTTTTTTAATTATTAAGAAGGAGAGCTAGCAGATACATTACCTC GTAATCGTGG-3′ (N-terminal) and 5′-ATCCCGCTCGAGTCATTTGTGTTTCTTTCCTC-3′ (C-terminal). The amplified product contained an XbaI restriction site (boldface) and ribosome binding site (underlined) preceding the initiation codon (italicized) of the bcTopo I gene (stop branch), 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 bcTopo I gene. Based on the published sequence, the ORF (3811119–3809131 of complete genome) that encodes bcTopo 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 3811144 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 bcTopo 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 bcTopo 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 III$\alpha$ and $bc$Topo I proteins

The induction of the $bc$Topo III$\alpha$ polypeptide was initiated by infection of the expression strain, harboring the pTopo3$\alpha$ plasmid DNA, with bacteriophage λC6. To prevent any contamination of the $bc$Topo III$\alpha$ protein with endogenous ecTopo III, it was purified from E.coli strain BL21 in which the gene encoding ecTopo 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 III$\alpha$ were then dialyzed overnight. The desalted crude protein extract was loaded onto 20 ml DE52 cellulose column (2.5 x 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 (20 mM Tris–HCl, pH 8.0; 1 mM DTT; 1 mM EDTA, pH 8.0; 40 mM Tris HCl buffer (pH 8.0 at 22°C) and 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 ecTopo I, ecTopo III, bcTopo I and bcTopo III$\alpha$ 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% 1x 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$. 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 ecTopo 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 III$\alpha$ ($bc$Topo III$\alpha$, gi: 30019974) and topoisomerase III$\beta$ ($bc$Topo III$\beta$, 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 ecTopo I. Similar to ecTopo I (30), it possesses three tetracysteine zinc finger motifs; however, the C-terminal region of ecTopo 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 ecTopo I,
bcTopo IIIα is composed of 714 amino acids with a molecular weight of 81.5 kDa and a predicted PI of 9.4. Among the first 600 amino acids, ecTopo III and bcTopo IIIα exhibit 32% identity and 55% similarity. Interestingly, unlike ecTopo III, bcTopo 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 bcTopo I and bcTopo IIIα, another type IA topoisomerase encoded by the B. cerasus genome is annotated bcTopo IIIβ. Although bcTopo IIIβ shares 35% sequence identity and 55% sequence similarity with ecTopo III, it is more similar to Bacillus subtilis topoisomerase III (64% sequence identity and 80% sequence similarity) based on sequence alignment (Z. Li and R. DiGate, manuscript submitted).

In order to characterize the biochemical activities of bcTopo I and bcTopo 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 λC6E phage. The bcTopo 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 bcTopo I and bcTopo IIIα have been assessed (Figure 3). Although both enzymes exhibit relaxation activity, their mechanism appears to be slightly different. bcTopo 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 high concentrations. In contrast, bcTopo 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 bcTopo I and bcTopo 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, Mg2+ and temperature characteristics of bcTopo I and IIIα have been determined based on a topoisomerase-mediated supercoiled DNA relaxation assay (Figure 4). bcTopo I exhibits consistent DNA relaxation activity within a pH range from 6.5 to 10.4 (Figure 4A), whereas, bcTopo 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 bcTopo IIIα is slightly inhibited at pH 7.5 and 10.4. Similar to ecTopo I (32), high concentrations of Mg2+ stimulate the DNA relaxation activity of bcTopo I (Figure 4C). Although the enzyme is able to relax DNA at 0.1 mM of Mg2+, it displays a distributive pattern rather than the processive pattern that appears at high concentrations of Mg2+. The optimal concentration of Mg2+ is 2.5 mM, higher concentrations of Mg2+ (up to 20 mM) slightly inhibit the DNA relaxation activity of bcTopo I. Unlike ecTopo III, whose DNA relaxation activity is inhibited by high concentrations of Mg2+ (>2 mM), bcTopo IIIα is capable of relaxing supercoiled DNA at a broad range of Mg2+ concentrations (Figure 4D). bcTopo IIIα also converts supercoiled DNA molecules into partially relaxed topoisomers that are close to that of the original supercoiled forms without addition of Mg2+ (Figure 4D, lane 2). This may be due to a trace amount of Mg2+ that remains during the protein purification process since the enzyme completely looses its relaxation activity in the presence of 1 mM EDTA. The optimal Mg2+ concentration...
5m MM g2
bc
bc
perature is needed for the optimal DNA relaxation activity of
37
bc
44, 22 and 0 fmol of
(pH 8.0 at 22
bc
2.5 mM Mg2
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tation activity. High temperature inhibits the DNA relaxa-
bc
cation described above.

To catalyze DNA relaxation, type IA topoisomerases need to
bc
bind and transiently cleave single-stranded DNA present in
for bcTopo IIIα-catalyzed DNA relaxation is between I and
10 mM. In addition, bcTopo IIIα is able to tolerate concentra-
tions of Mg2++ up to 20 mM with only a slight decrease in
relaxation activity. High temperature inhibits the DNA relaxa-
tion activity of bcTopo I (Figure 4E). In contrast, high tem-
perature is needed for the optimal DNA relaxation activity of
bcTopo IIIα (Figure 4F). The optimal reaction temperature for
bcTopo IIIα-catalyzed DNA relaxation is 52°C, the same as
that of ecTopo III. Temperatures >37°C reduce the activity of
bcTopo I.

bcTopo I and bcTopo 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 bcTopo I and bcTopo
IIIα (Figure 5A). A 28 base 32P-labeled oligonucleotide con-
taining ecTopo III cleavage sites was used as the substrate.
Although ecTopo I and bcTopo I possess three Zinc finger
motifs, bcTopo I does not have an extra C-terminal region
present in ecTopo I. ecTopo I and bcTopo 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 ecTopo I C-terminal region may not be critical
for single-stranded DNA binding.

ecTopo III and bcTopo 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, bcTopo 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 bcTopo I, bcTopo IIIα,
ecTopo I and ecTopo III (Figure 5B). bcTopo I and ecTopo
I share three common cleavage sites but the enzymes prefer
different subsets of the cleavage sites. bcTopo IIIα and ecTopo
III also exhibit common cleavage sites with different prefer-
ces. bcTopo I and bcTopo IIIα do not share the same major
cleavage sites using this substrate. Besides supercoiled DNA
relaxation efficiency, the single-stranded DNA cleavage spe-
cificity may also be considered as another property that dis-
tinguishes ecTopo I-like topoisomerase from ecTopo III-like
enzymes.

Based on the X-ray crystal structure of ecTopo 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
ecTopo III-like enzymes reveals that residues of this region
are generally conserved (Figure 6). Interestingly, the corre-
sponding regions in ecTopo I-like enzymes are virtually
identical. Furthermore, several conserved residues present
in ecTopo III-like proteins are different from those present
in ecTopo 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.

bcTopo IIIα is a potent DNA decatenase,
but not bcTopo I

ecTopo 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,
have been shown to play an essential role in ecTopo III-
catalyzed DNA decatenation and termed as ‘decatenation
loop’ (9). Many ecTopo III-like enzymes also possess the
decatenation loop and exhibit DNA decatenation activity.
bcTopo IIIα also possesses the decatenation loop and it shares
similar biochemical properties with ecTopo III. bcTopo IIIα

Figure 3. (A) DNA relaxation assay of bcTopo I. Reactions (25 µl) contained
5 mM Mg2+, 1 mM DTT, 30% glycerol, 100 ng/µl BSA and 40 mM Tris–HCl
(pH 8.0 at 22°C) with indicated amount of bcTopo I. Lanes 1–6: 312, 166, 88,
44, 22 and 0 fmol of bcTopo I, respectively. The reactions were incubated at
37°C for 30 min. The reaction products were electrophoresed through a 1% 1x
TAE agarose gel and the DNA topoisomeras were visualized by ethidium bro-
mide staining. (B) DNA relaxation assay of bcTopo IIIα. Lanes 1–5: 1.25, 0.25,
0.05, 0.01 and 0 pmol of bcTopo IIIα, respectively. The reactions (containing
2.5 mM Mg2+ 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.
Figure 4. (A) Effect of pH on bcTopo I-mediated DNA relaxation. Reactions (25 μl) contained 125 fmol of bcTopo I, 2.5 mM Mg<sup>2+</sup> 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 III<sub>α</sub>-mediated DNA relaxation. Reactions (25 μl) contained 125 fmol of bcTopo III<sub>α</sub>, 2.5 mM Mg<sup>2+</sup> 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<sup>2+</sup> concentration on bcTopo I-mediated DNA relaxation. Reactions were performed as described above except with different Mg<sup>2+</sup> concentrations. Lanes 1–7: 0, 0.1, 1, 2.5, 5, 10 and 20 mM Mg<sup>2+</sup>, respectively. Lane 8, no protein. (D) Effect of Mg<sup>2+</sup> concentration on bcTopo III<sub>α</sub>-mediated DNA relaxation. Lane 1, 1 mM of EDTA without Mg<sup>2+</sup>; lanes 2–8, 0, 0.1, 1, 2.5, 5, 10 and 20 mM Mg<sup>2+</sup>, respectively; Lane 9, no protein. Reactions were performed as described above except with different Mg<sup>2+</sup> concentrations. (E) Effect of temperature on bcTopo 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 bcTopo III<sub>α</sub>-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 \textit{bc} Topo III, \textit{ec} Topo III, \textit{bc} Topo I and \textit{ec} Topo I. The assay was performed as described in Materials and Methods. Reactions (10 µl) contained 5 pmol of \textit{32}P-radiolabeled oligonucleotide, 1 mM Mg\textsuperscript{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 \textit{ec} Topo III; lanes 5–7: 0.4, 1.1 and 3.3 pmol of \textit{bc} Topo III; lanes 8–10: 0.4, 1.1 and 3.3 pmol of \textit{bc} Topo I a; lanes 11–13, 0.4, 1.1 and 3.3 pmol of \textit{ec} Topo I. The reactions were electrophoresed through 12% polyacrylamide gel in 0.5x TBE and visualized by autoradiography. (B) Comparison of the single-stranded DNA cleavage site specificities of \textit{bc} Topo III, \textit{ec} Topo III, \textit{bc} Topo I and \textit{ec} Topo I. The assay was performed as described in Materials and Methods. Reactions (10 µl) contained 5 pmol of \textit{32}P-radiolabeled oligonucleotide, 1 mM Mg\textsuperscript{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 \textit{ec} Topo III, respectively; lanes 5–7: 1.1, 3.3 and 10 pmol of \textit{bc} Topo III, respectively; lanes 8–10: 1.1, 3.3 and 10 pmol of \textit{ec} Topo I, respectively; lanes 11–13: 1.1, 3.3 and 10 pmol of \textit{bc} Topo I, respectively. The reactions were electrophoresed through a 15% polyacrylamide gel in 1x TBE with 50% urea and visualized by autoradiography. The common cleavage sites of \textit{bc} Topo III, \textit{ec} Topo III, \textit{bc} Topo I and \textit{ec} Topo I are indicated by right arrow; The common cleavage sites of \textit{bc} Topo I and \textit{ec} Topo I are indicated by left arrow; unique cleavage sites created by \textit{bc} Topo III a are indicated by dash; unique cleavage sites created by \textit{ec} Topo III, \textit{bc} Topo I and \textit{ec} Topo I are indicated by dots.
is able to resolve oriC based plasmid DNA replication intermediates in vitro (Figure 7). Although bcTopo I is very efficient at the relaxation negatively supercoiled DNA, like ecTopo I, it is not an efficient decatenase. This implies that different catalytic mechanisms have been applied by bcTopo I and bcTopo IIIα to perform intramolecular (which results in relaxation) and intermolecular (which results in decatenation) strand passage.

bcTopo I and bcTopo IIIα are able to substitute for the loss of ecTopo III in vivo

It has been proposed that ecTopo I in conjunction with DNA gyrase regulates superhelical density in E.coli. Therefore, cells with a deletion of topA (the gene encoding ecTopo 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 ecTopo III protein for cells to survive.

If bcTopo I and bcTopo IIIα can perform similar functions as their E.coli counterparts in vivo, bcTopo IIIα should be able to substitute for the loss of ecTopo 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 bcTopo I (pTopo1) and bcTopo 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:kanrt; however, cells harboring either pDE1, pBT3α and pBT1 can be transduced to topB:kanrt (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. perfringens, C. tetani, E. faecalis* and *S. epidermidis* also contain three chromosome copies of type IA topoisomerases that exhibit extensive amino acid sequence similarity with bcTopo I, bcTopo IIIα and bcTopo 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 bcTopo 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
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. bcTopo I shares extensive sequence similarity with ecTopo 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 ecTopo I) and it also lacks a highly positively charged N-terminal loop (residues 41–69) present in ecTopo I. Since bcTopo I and ecTopo I exhibit similar biochemical properties, it appears that these regions may not be critical for the catalytic activity of bcTopo I but may relate to the specific biological function of ecTopo I in vivo (e.g. by mediating protein–protein interactions). bcTopo I is efficient in relaxing negatively supercoiled DNA in a processive manner. The DNA relaxation activity of bcTopo I, similar to ecTopo I, is stimulated by high concentration of Mg2 and is inhibited by high temperature. In addition, bcTopo I and ecTopo I display comparable single-stranded DNA binding activities and share common DNA cleavage sites. Similar to ecTopo I, bcTopo I is not capable of resolving multiple interlinked DNA dimers.

bcTopo IIIα and ecTopo III exhibit extensive protein sequence homology within the first 600 amino acids; however, there is a little homology within their C-terminal sequence. bcTopo 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 bcTopo IIIα tends to form a unique supershift complex with single-stranded oligonucleotides when compared with ecTopo III. In addition, bcTopo IIIα, unlike ecTopo III, is able to relax supercoiled DNA under a broad range of Mg2 concentrations ranging from a trace amount to 20 mM. Overall, however, bcTopo IIIα is clearly an ecTopo III-like enzyme. Similar to ecTopo III, it is inefficient at the relaxation of negatively supercoiled DNA and the activity is stimulated by high temperature. bcTopo IIIα and ecTopo III also exhibit similar single-stranded DNA cleavage specificity and more importantly, bcTopo IIIα is a potent decatenase, a characteristic that is typical of ecTopo III-like enzyme.

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

Although bcTopo I and bcTopo IIIα exhibit distinctly different biochemical properties, they are both able to substitute for the loss of ecTopo III in E.coli DM750 cells. This is distinct contrast to the results with ecTopo I. The inability of ecTopo I to compensate for the loss of ecTopo 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

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**Figure 7.** Resolution of the oriC replication intermediates by bcTopo I and bcTopo IIIα. The reactions (12.5 µl) contained no protein (lanes 1 and 6); 17.5 fmol of bcTopo I or bcTopo IIIα (lanes 2 and 6); 35 fmol of bcTopo I or bcTopo IIIα (lanes 3 and 7); 70 fmol of bcTopo I or bcTopo IIIα (lanes 4 and 8); and 140 fmol of bcTopo I or bcTopo 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

| Organism | Plasmid | Transduction frequency (×10<sup>-3</sup>) |
|----------|---------|------------------------------------------|
| E.coli K12 | pDE1 | 0.36 |
| E.coli K12 | pT11 | N/A |
| B.anthracis | pBT3α | 0.63 |
| B.anthracis | pBT1 | 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::kanr 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 (20) |
| E.coli K12 | topA | 865 | 122 (4) |
| B.anthracis | topBα | 714 | 340 (20) |
| B.anthracis | topBβ | 729 | 320 (20) |
| B.anthracis | pXO2-77/76α | 676 | 289 (34) |
| B.anthracis | pXO1-142α | 887 | 199 (32) |
| B.anthracis | topA | 692 | 105 (20) |
| B.cereus | topBα | 714 | 337 (21) |
| B.cereus | topBβ | 729 | 328 (21) |
| B.anthracis | 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 putative topoisomerase encoded by pXO2 plasmid was formed by changing the amber stop codon TAG of gene 77 to GAG.

* TopX encoded by pXO1 gene 142.
the loss of ecTopo I activity in the cell (6,7). Introduction of the wild-type gene encoding ecTopo 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 bcTopo I and ecTopo I is very similar over a broad range of pH and salt concentration (data not shown). It is quite probable that both bcTopo I and ecTopo 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 ecTopo I in a process other than the maintenance of superhelical density (37).

Interestingly, similar to the results with ecTopol, bcTopo 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 ecTopo I is not strictly unique to this enzyme but has its basis in a true functional deficiency. bcTopo IIIβ is a unique topoisomerase that is different from ecTopo 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.perfringens, Enterococcus faecalis 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 ecTopo 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 conjugal 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.

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