Published in final edited form as:

J Mol Biol. 2009 January 16; 385(2): 558–567. doi:10.1016/j.jmb.2008.10.073.

Asp to Asn substitution at the first position of the DxD TOPRIM motif of recombinant bacterial topoisomerase I is extremely lethal to *E. coli*

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SUMMARY

The TOPRIM domain found in many nucleotidyl transferases contains a DxD motif involved in magnesium ion coordination for catalysis. Medium to high copy number plasmid clones of *Yersinia pestis* topoisomerase I (YpTOP) with Asp to Asn substitution at the first aspartate residue (D117N) of this motif could not be generated in *Escherichia coli* without second site mutation even when expression was under the control of the tightly regulated BAD promoter and suppressed by 2% glucose in the medium. Arabinose induction of a single copy YpTOP-D117N mutant gene integrated into the chromosome resulted in $\sim 10^5$ fold of cell killing in 2.5 h. Attempt to induce expression of the corresponding *E. coli* topoisomerase I mutant (EcTOP-D111N) encoded on a high copy number plasmid resulted in either loss of viability or reversion of the clone to wild-type. High copy plasmid clones of YpTOP-D119N and EcTOP-D113N with the Asn substitution at the second Asp of the TOPRIM motif could be stably maintained, but overexpression also decreased cell viability significantly. The Asp to Asn substitutions at these TOPRIM residues can selectively decrease Mg^{2+} binding affinity with minimal disruption of the active site geometry, leading to trapping of the covalent complex with cleaved DNA and causing bacterial cell death. The extreme sensitivity of the first TOPRIM position suggested that this might be a useful site for binding of small molecules that could act as topoisomerase poisons.

Keywords

topoisomerase; TOPRIM; DNA cleavage; DNA religation; bactericidal

Introduction

Bacterial DNA topoisomerase I belongs to the type IA family of DNA topoisomerase. This class of enzyme removes excess negative supercoiling from chromosomal DNA by cleaving the single-stranded region of negatively supercoiled DNA while forming a covalent complex with the cleaved DNA, followed by passage of a second single DNA strand through the break,

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and rejoining of the cleaved DNA strand. Topoisomerase poisons targeting type IB and type IIA topoisomerases are effective antibacterial and anti-cancer agents because these drugs can inhibit the DNA rejoining step of these topoisomerases¹⁻³. These drugs lead to the accumulation of the covalent topoisomerase-cleaved DNA complex to initiate cell killing. Bacterial topoisomerase I should also be a susceptible target for discovery of novel antibacterial compounds that act by a similar mechanism⁴. It has been demonstrated that accumulation of bacterial topoisomerase I covalent complex in *Escherichia coli* due to a mutation in the topoisomerase I coding sequence will indeed lead to rapid cell killing⁵. Characterization of these topoisomerase I mutant proteins that accumulate the covalent cleaved complex in vivo provide information on how the DNA cleavage-religation equilibrium of type IA topoisomerases can be perturbed and could be very useful for the drug discovery effort. Small molecules when bound to the topoisomerase I molecule can potentially mimic the effect of the mutation to initiate bactericidal pathway due to the accumulation of the covalent topoisomerase complex.

The first such mutation characterized in bacterial topoisomerase I was identified by screening of a library of mutagenized recombinant *Yersinia pestis* topoisomerase I (YpTOP) clones for the ability to induce the SOS response of *E. coli* due to DNA damage⁵. It was found that induction of YpTOP-G122S expressed under the control of the arabinose-inducible BAD promoter on a high copy number plasmid can result in 4 to 5 log decrease in viable counts in 2 h. This Gly to Ser substitution is at the TOPRIM Dx₁DxxG motif conserved for divalent ion interaction among families of nucleotidyl transferases⁶. A new recombinant YpTOP mutant with a D117N substitution along with other mutations has been identified from the mutagenized YpTOP library with the SOS induction screen in *E. coli*. Attempts to construct *Y. pestis* or *E. coli* topoisomerase I clones containing only this Asp to Asn substitution at the first position of the TOPRIM motif showed that this mutation has a much more severe lethal effect than the Gly to Ser substitution. Medium to high copy number clones of YpTOP-D117N could not be isolated in *E. coli* without the presence of second site mutation even when expression from the BAD promoter was suppressed with 2% glucose. The experiments reported here demonstrate that the DNA religation activity of bacterial topoisomerase I can be selectively inhibited while maintaining the DNA cleavage activity by directly interfering with the Mg²⁺ coordination at the active site.

Results

Isolation of an SOS inducing recombinant YpTOP mutant with the D117N mutation

We wish to identify sites on the bacterial topoisomerase I that are critical for the efficient religation of DNA after DNA cleavage and strand passage during the catalytic cycle. Randomly mutagenized recombinant YpTOP clones under the control of the BAD promoter were screened for the ability to induce the SOS response of *E. coli* after induction of the mutant YpTOP protein with a low level of arabinose (0.002%). The clones were normally maintained with the expression repressed by the presence of 2% glucose in the medium as accumulation of the cleavage complex formed by topoisomerase I would be potentially lethal. One of the SOS-inducing clones identified, pYTOP39, was found to contain the D80N, G94S and D117N mutations. Induction of this mutant YpTOP expression with 0.2% arabinose in JD5 resulted in extensive bacterial cell death (Table 1).

High copy number plasmid expressing YpTOP-D117N could not be maintained in *E. coli* without a second site mutation

Asp117 of YpTOP is at the first position of the TOPRIM residues Dx₁D responsible for coordination of divalent metal ions in type IA and type IIA topoisomerases as well as many other nucleotidyl transferases^{6, 7}. We therefore postulated that the D117N mutation in

YpTOP39 might lead to deficiency in DNA religation. Attempts were made to introduce the D117N mutation as the single substitution in plasmid pYTOP. Sequencing of the clones isolated in *E. coli* TOP10 after the site directed mutagenesis procedures showed that the D117N mutation was always associated with a second site mutation even though the clones were isolated and maintained in the presence of 2% glucose to suppress the BAD promoter. The *E. coli* TOP10 strain had a *recA* genotype which conferred hypersensitivity to the topoisomerase I cleavage complex⁸. Attempts were therefore made to isolate the pYTOP-D117N clone after mutagenesis by transformation of the *E. coli* BL21 strain which is *recA*⁺. The clones isolated from BL21 similarly also had second site mutations. Many of the second site mutations resulted in frame shift or early terminations. Transformations of these clones into *E. coli* JD5 with the *dinD::lacZ* fusion resulted in white colonies on X-gal plate with arabinose indicating no DNA damage due to the expressed mutant proteins. However, some of the clones with a second amino acid substitution in addition to the D117N mutation when transformed into *E. coli* JD5 gave rise to blue colonies on X-gal plate with arabinose and overexpression of these YpTOP proteins resulted in significant loss of viability (Table 1). This suggested that these second site mutations only partially suppressed the lethal effect of the D117N mutation. *E. coli* topoisomerase I also has three tetra-cysteine zinc binding motifs and previous biochemical studies have shown that zinc coordination is required for cleavable complex formation with DNA⁹. Several of these second site suppression mutations included in Table 1 are found in the tetra-cysteine zinc binding motifs of YpTOP.

Attempt was also made to express YpTOP-D117N under the control of the T7 promoter in a plasmid constructed by ligation independent cloning¹⁰. A mutant clone pLIC-YTOP-D117N was successfully isolated in *E. coli* strain NEB Turbo (from New England BioLabs) in LB medium with 2% glucose. However, the clone could not be transformed into any *E. coli* strain with T7 RNA polymerase present even when T7 RNA polymerase activity was suppressed by T7 lysozyme¹¹ or when T7 RNA polymerase expression under the control of the BAD promoter (in strain BL21AI from Invitrogen) was repressed in the presence of 2% glucose. When NEB Turbo cells transformed with LIC-YTOP-D117N was grown in LB medium with 1 mM MgSO₄ to prepare for infection by lambda phage CE6 in order to supply the T7 RNA polymerase, growth halted at around OD₆₀₀=0.2. Only mutant proteins with deletions as determined by SDS gel electrophoresis of the total proteins were found to be expressed after the phage infection (data not shown).

The SOS-inducing YpTOP-D117N,C737Y mutant topoisomerase is enhanced in DNA cleavage product formation

We purified YpTOP-D117N,C737Y mutant protein for further characterization because it could be expressed and purified in relatively large yield from *E. coli* JD5 after induction with 0.02% arabinose. It had no relaxation activity (Figure 1A). For wild-type YpTOP, DNA cleavage did not require addition of Mg²⁺ to the reaction mixture, and the addition of 2 mM Mg²⁺ reduced the level of cleavage product observed because of Mg²⁺ dependent religation of the cleavage product (Figure 1B). Cleavage product could not be observed for YpTOP-D117N,C737Y in the absence of added Mg²⁺. In the presence of 2 and 5 mM MgCl₂, the level of cleavage product formed by YpTOP-D117N, C737Y was around 3-5 fold higher than that of wild-type YpTOP. The increased level of DNA cleavage product formed in the presence of Mg²⁺ is likely to be responsible for the SOS-inducing and cell killing phenotypes of the YpTOP mutants with the D117N mutation (Table 1). Cys737 corresponds to the third cysteine in the last of the three tetracysteine zinc binding motifs found in YpTOP. The overexpression of YpTOP-C737Y in JD5 did not induce the SOS response, and the overexpression had no effect on the viability (Table 1). Analysis of purified YpTOP-C737Y showed that the C737Y mutation is a novel type of mutation that resulted in faster religation rate and reduced relaxation activity when compared to wild-type YpTOP (Cheng & Tse-Dinh, to be published). The faster

religation rate from the C737Y mutation probably accounted for the selection of the YpTOP-D117N, C737Y mutant for suppression of the lethal effect of the D117N mutation during site-directed mutagenesis to introduce the D117N mutation into YpTOP.

YpTOP-D117N in single or very low copies is much more lethal than YpTOP-G122S

We were able to introduce the D117N mutation into the YpTOP gene under the control of the BAD promoter in a low copy number pBRINT-T_sCM plasmid designed for integration into the chromosome¹². Maintenance of this plasmid at the lower temperature of 30°C probably reduced the background transcription of the YpTOP gene. We have previously observed that lower level of background transcription of toxic recombinant vaccinia virus topoisomerase I gene from a high copy number plasmid occurred at 30°C when compared to 42°C¹³. This low copy number clone pBRYTOP-D117N allowed us to study the effect of the single D117N mutation on cell viability but mutant YpTOP protein expression level was very low and detectable only by western blot and not by Coomassie blue staining of the total proteins (Figure 2). This was not just due to the low copy number of the plasmid, but also because induction of the BAD promoter on plasmid pYTOP at 30°C was less efficient than at 37°C (Sutherland and Tse-Dinh, unpublished results). Integration of plasmid pBRYTOP wild-type and mutant derivatives into the *lacZ* gene region of *E. coli* chromosome was first achieved in MG1655 to construct the strains MGYTOP (wild-type YpTOP integrated strain), MG122S and MG117N. The low copy number pBRYTOP-G122S at 30°C or single copy integrated YpTOP-G122S in MG122S at 37°C had minimal effect on viability (Table 2). Viability measurement after induction of the YpTOP-D117N present in single or very low copies (Table 2) demonstrated that the D117N mutation is ~200-1000 times more lethal than the previously characterized G122S mutation, even though the level of YpTOP-G122S mutant protein expression appeared to be higher when analyzed by western blot (Figure 2A). The lower level of accumulated YpTOP-D117N mutant protein could be due to its toxicity. The *E. coli* strain BW27784 with the arabinose transporter gene *araE* under the control of a constitutive promoter allowed uniform concentration dependent induction of the BAD promoter by a range of arabinose concentrations¹⁴. In contrast, uniform induction of the BAD promoter in strain MG1655 requires saturating concentration (0.2%) of arabinose. P1 phage transduction was used to move the integrated YpTOP and YpTOP-D117N gene from MGYTOP and MG117N respectively into the chromosome of *E. coli* strain BW27784 to construct the strains BWYTOP, BW117N. Western blot analysis was used to correlate the expression of the YpTOP-D117N protein from the chromosome (Figure 2B) and the loss of viability (Table 2). Saturating level of YpTOP-D117N protein expression and cell killing was achieved with 0.02% arabinose.

Expression of YpTOP-D119N but not YpTOP-E121Q from high copy number plasmid could induce the SOS response and result in bacterial cell death

In bacterial topoisomerase I sequences, there is a third conserved acidic residue within the TOPRIM motif (DXDXEG). The two Asp residue along with the Glu residue form the acidic triad in the active site previously shown to be involved in binding of two Mg²⁺⁷. Site directed mutagenesis of the YpTOP Asp119 residue to Asn and Glu121 to Gln using pYTOP as template were successful, resulting in clones with no other second site mutation. Strain JD5 transformed with pYTOP-D119N formed a blue colony on Xgal plate containing 0.002% arabinose, indicating induction of the SOS response. In contrast, SOS induction was not observed with the YpTOP-E121Q protein. The stability of the pYTOP-D119N clone showed that the D119N mutation was not as lethal as the D117N mutation. From measurement of the cell killing effect in JD5 with 0.2% arabinose induction, the D119N mutation had significantly more lethal effect than the E121Q mutation (Table 1).

YpTOP-D119N protein has reduced relaxation activity and DNA cleavage required added Mg^{2+}

YpTOP-D119N and YpTOP-E121Q proteins were purified after induction with 0.02% arabinose in *E. coli* strain JD5. Relaxation assays of serial dilutions of wild-type YpTOP and YpTOP-D119N (Figure 3A) showed that the D119N mutation resulted in >5 fold loss relaxation activity (from comparison of relaxation activity of 5 ng of wild-type YpTOP versus 25 ng of YpTOP-D119N) with only partially relaxed DNA products from incubation with YpTOP-D119N. Further quantitation of results from time course of relaxation by densitometry¹⁸ using 100 ng of the enzymes (Figure 3B) showed that the initial rate of relaxation is ~9 times slower for YpTOP-D119N. Results from DNA cleavage assay (Figure 3C) showed that similar to YpTOP-D117N,C737Y, DNA cleavage was not observable without Mg^{2+} added to the reaction. The E121Q mutation resulted in greater decrease of the relaxation activity (>20 fold, Figure 3A). More significantly, DNA cleavage of oligonucleotide substrate was not readily observable even in the presence of added Mg^{2+} . The lack of SOS induction and cell killing, as well as decrease in relaxation activity, by the YpTOP-E121Q mutant is thus likely due to the severely reduced DNA cleavage activity.

Enhanced DNA cleavage product formation by EcTOP67-D111N

Asp111 and Asp113 are the two aspartate residues in the TOPRIM motif of *E. coli* topoisomerase I (EcTOP). Attempts were made to express the full length EcTOP-D111N mutant protein under the control of either the BAD promoter or T7 promoter. Although clones could be isolated in NEB Turbo cells in LB medium in the presence of 2% glucose, growth of the clones in medium without glucose would cease in the early exponential phase, and plasmid isolated from the induced cultures showed that the clones had reverted back to wild-type EcTOP sequence. The 67 kD N-terminal fragment (EcTOP67) of EcTOP corresponds to the conserved transesterification domain. It has no relaxation activity, but retained DNA cleavage activity¹⁵. To determine the effect of the mutation on DNA cleavage-religation, mutant EcTOP67-D111N was expressed under the control of the T7 RNA polymerase. It required added Mg^{2+} for formation of the DNA cleavage product from the 59 base oligonucleotide substrate (Figure 4A). Based on quantitation of 5 sets of data by densitometry, in the presence of 2 mM Mg^{2+} , the level of DNA cleavage product formed by EcTOP67-D111N was 18 fold higher than that of wild-type EcTOP67 and 6.8 fold higher than EcTOP67-G116S. The level of DNA cleavage product formed by wild-type EcTOP67 from this 59 base oligonucleotide substrate was too low for analysis of DNA religation. A shorter 13 base DNA oligonucleotide gave higher amount of DNA cleavage product from wild-type EcTOP67 with no Mg^{2+} added, while the level of cleavage product observed at 2 mM Mg^{2+} was 11 fold higher for EcTOP67-D111N in the comparison with EcTOP67 (Figure 4B). Analysis of DNA religation with this 13 base oligonucleotide substrate (Figure 4C) showed that for wild-type EcTOP67, DNA religation already reached the maximum level after 10 s at 0°C, but the rate was significantly slower for EcTOP67-D111N. Therefore even though attempts to purify full length bacterial topoisomerase I with the single Asp to Asn mutation at the first TOPRIM position was unsuccessful, results from the EcTOP67 transesterification domain indicated that this mutation greatly enhanced the level of the DNA cleavage product while inhibiting DNA religation. The high level of cleavage product accumulated in vivo probably accounted for the extreme lethal effect of this substitution.

Characterization of EcTOP-D113N enzymatic activity and Mg^{2+} binding affinity

Unlike full length EcTOP-D111N, EcTOP-D113N mutant protein was successfully expressed and purified. Characterization of the relaxation activity showed up to 100 fold reduction in relaxation activity compared to wild-type EcTOP (Figure 5A). Cleavage of the 59 base oligonucleotide substrate could not be observed in the absence of added Mg^{2+} (Figure 5B).

While religation of the DNA cleavage product by wild-type EcTOP was >90% complete in 30s in the presence of Mg^{2+} , religation by EcTOP-D113N proceeded at a much slower rate (Figure 5C). The requirement of added Mg^{2+} for DNA cleavage and reduced rate of DNA religation correlated with a lower affinity for Mg^{2+} measured by change in tryptophan fluorescence upon Mg^{2+} binding (Figure 6). Each molecule of wild-type EcTOP has been shown previously by ICP analysis to bind two Mg^{2+} ¹⁶. Using the GraphPad Prism software, the data from wild-type EcTOP can be best fitted to two binding sites ($K_{d1}=1.3 \mu M$, $K_{d2} = 75.2 \mu M$)¹⁷. Curve fitting of the data from three sets of measurements gave the values of $K_{d1}=7.5 \mu M$, $K_{d2} = 308.8 \mu M$ for EcTOP-D113N.

Discussions

DNA cleavage by *E. coli* topoisomerase I does not require Mg^{2+} to be added to the reaction mixture¹⁸. However, Mg^{2+} must be added for the DNA religation step of the catalytic cycle and for the overall relaxation of negatively supercoiled DNA^{19, 20}. For wild-type *E. coli* and *Y. pestis* topoisomerase I, the presence of Mg^{2+} reduced the level of oligonucleotide DNA cleavage product observed because of DNA religation. Interaction with the two Mg^{2+} ions in the active site is thus critical for the DNA cleavage-religation equilibrium and has emerged in the characterization of the SOS-inducing *Y. pestis* topoisomerase I mutants described here and in previous studies^{5, 21} as the key factor for the level of accumulated DNA cleavage product.

The strong influence of the TOPRIM DXDXXG motif on DNA cleavage and religation by bacterial type IA topoisomerase was first demonstrated by the inhibition of DNA religation by the Gly to Ser mutation⁵. This mutation was the only substitution at that position that retained significant cleavage activity while inhibiting DNA religation¹⁷. The slightly larger side chain of Ser versus the very small Gly side chain probably distorted the folding of the TOPRIM domain sufficiently to reduce Mg^{2+} binding affinity and inhibited DNA religation. The level of cleavage product formed by this mutant enzyme however was lower than that of wild-type topoisomerase I, limiting the level of covalent complex accumulation and cell killing unless DNA cleavage was enhanced by a second mutation not at the TOPRIM domain but at the methionine residue adjacent to the active site tyrosine¹⁷. It was also possible to interfere with Mg^{2+} binding at the bacterial topo I active site by introduction of an additional positive charge via the methionine to arginine mutation adjacent to the active site tyrosine²¹. This Met to Arg mutation did not inhibit religation completely, but the level of DNA cleavage product formed by the YpTOP-M326R mutant was significantly higher than that of the YpTOP-G122S. The key to the bactericidal effect is the slowing of the rate of DNA religation while maintaining a robust DNA cleavage activity in the presence of Mg^{2+} ions so that the covalent cleavage complex would accumulate on the chromosome. The conservative single substitution of one of the Mg^{2+} binding aspartates in the TOPRIM motif with asparagine is thus expected to have a high probability of affecting Mg^{2+} binding and DNA religation with minimal disruption of the active site structure for DNA cleavage. One of the SOS inducing YpTOP mutants isolated after random mutagenesis of recombinant YpTOP gene did indeed possess the D117N substitution. However, even with the expression under the BAD promoter tightly repressed by 2% glucose, YpTOP mutant with this substitution could only be isolated on medium to high copy number plasmids with other mutations present in the same clone that suppressed the extreme lethal effect of the D117N substitution. Western blot analysis along with viability measurement showed that by removing a single Mg^{2+} coordinating negative charge, this D117N substitution is up to 1000 fold more lethal than the TOPRIM G122S mutation that inhibited Mg^{2+} binding by structural perturbation. Unlike the full length enzyme, the 67kDa EcTOP67 N-terminal fragment with the D111N substitution could be expressed successfully likely because the absence of the C-terminal domain reduced both the DNA binding affinity²² and interaction with RNA polymerase²³. The level of DNA cleavage product formed by EcTOP67-D111N protein was found to be >11 fold higher than that of wild-type EcTOP67

(Figure 4A,B). The much higher level of DNA cleavage product is likely the reason behind the extreme lethal effect of the bacterial topoisomerase I mutant enzyme with the Asp to Asn substitution at the first Asp of the TOPRIM motif.

The Asp to Asn substitution at the second Asp of the TOPRIM motif was not as lethal as the substitution at the first Asp, but overexpression of YpTOP-D119N mutant also resulted in up to 10^5 fold loss of viability when compared to the non-induced culture. A lower Mg^{2+} binding affinity corresponded to reduced rate of DNA religation, and higher level of DNA cleavage product when compared with the wild-type enzyme. In contrast, the Glu to Gln substitution at the third position of the acidic triad within the bacterial topoisomerase I TOPRIM motif DXDXEG resulted in much lower level of DNA cleavage product (Figure 3C), accounting for the relatively small effect of overexpression of the YpTOP-E121Q mutant on viability (Table 1). Therefore the loss of each of the three Mg^{2+} coordinating carboxylates had a different effect on the DNA cleavage-religation equilibrium, with the Asn substitution at the first Asp favoring accumulation of the DNA cleavage product, and Glu to Gln substitution at the third position reducing the level of DNA cleavage product. The Asp to Asn substitution at the second carboxylate reduced the rate of DNA religation while increasing the Mg^{2+} dependence for DNA cleavage (Figure 5). This may be due to the two Mg^{2+} coordinated by the acidic triad contributing differently to DNA cleavage and religation. Based on the position of the acidic triad in the active site of *E. coli* topoisomerase I (Figure 7), the Mg^{2+} could assist the phosphoryl transfer by several mechanisms including activation of the tyrosine hydroxyl nucleophile, positioning the scissile phosphate and stabilizing the transition state or leaving group²⁴. It has been shown here and also in previous reports^{18, 26} that Mg^{2+} does not have to be added to observe DNA cleavage by wild-type EcTOP. At the end of purification, the enzyme was dialyzed into storage buffer containing 0.2 mM EDTA for 24 h with one change of buffer. The oligonucleotide cleavage reaction mixture also contained 1 mM EDTA to chelate any divalent ion present in the solution in low concentration. Nevertheless, it cannot be ruled out that the wild-type EcTOP and YpTOP preparations have a divalent ion bound at the high affinity site, with the off rate for this bound divalent ion being extremely slow.

Conclusions

The various SOS-inducing YpTOP mutants have demonstrated that bacterial topoisomerase I could be turned into a cell killing molecule by affecting the Mg^{2+} -enzyme interaction at the active site. This was accomplished effectively by removing a specific negative charge responsible for Mg^{2+} coordination. The extreme lethal effect of the Asp to Asn substitution at the first TOPRIM position showed that this is the most sensitive site. Small molecules that could inhibit or compete for Mg^{2+} binding in the active site should be useful as leads for novel topoisomerase poisons in the discovery of antibacterial compounds.

Materials and Methods

Random mutagenesis and screening of YpTOP

Recombinant YpTOP was cloned under the control of the BAD promoter in plasmid pYTOP⁵ derived from pBAD/Thio (Invitrogen). After random mutagenesis of the YpTOP coding sequence by the GeneMorph II EZClone domain mutagenesis kit (Stratagene), the pYTOP mutant plasmid library⁵ was transformed into *E. coli* JD5 with the *dinD::lacZ* fusion²⁵. The transformants were first isolated on LB plates with 100 µg/ml ampicillin and 2% glucose. The colonies were then replica plated onto LB plates with ampicillin, 0.002% arabinose and 35 µg/ml Xgal to identify mutant YpTOP clones that induce the synthesis of β-galactosidase (indicated by blue colored colony phenotype) from the *dinD* promoter by activating the RecA mediated SOS response to DNA damage. Plasmid clone pYTOP39 was isolated from a blue colony on the Xgal plate.

Site-directed mutagenesis and mutant protein purification

Site-directed mutagenesis of individual residues on YpTOP and EcTOP was carried out with Pfu Ultra II Fusion HS DNA polymerase and the QuikChange site-directed mutagenesis protocol (Stratagene). Sequence of the entire topoisomerase coding region was determined prior to and during expression to monitor occurrence of reversion or second site suppression mutations. Expression of individual mutant YpTOP proteins under the control of the BAD promoter on derivatives of plasmid pYTOP in *E. coli* JD5 was induced by adding 0.02% arabinose to exponential phase culture. Cells were harvested after 3 h at 37°C. The YpTOP proteins with the 6x-His tag at the C-terminus were purified using the nickel-nitrilotriacetic acid affinity column (Qiagen) according to the manufacturer's protocol.

The wild-type EcTOP 67 kD N-terminal fragment and its D111N or G116S mutant derivatives were cloned and expressed under the control of the T7 promoter in *E. coli* BL21AI strain with tight regulation of the T7 RNA polymerase by the BAD promoter (Invitrogen) and purified as described previously for the full length EcTOP²¹.

Expression of EcTOP-D113N under the control of T7 promoter on plasmid pLIC-ETOP derivative in *E. coli* BL21 was achieved by infection with lambda CE6 phage to introduce the T7 RNA polymerase¹¹.

Purified proteins were dialyzed into storage buffer of 0.1 M potassium phosphate, pH 7.4, 0.2 mM dTT, 0.2 mM EDTA and 50% glycerol.

Integration of genes encoding recombinant *Y. pestis* topoisomerases into *E. coli* chromosome

Chromosomal integration of mutant or wild type *Y. pestis topA* was accomplished using a chloramphenicol-resistant pBRINT-T_sCM vector system¹² containing a temperature-sensitive replicon designed for chromosomal integration of the desired genes into the *lacZ* gene of *E. coli*. The DNA fragment for synthesizing recombinant wild-type *Y. pestis* topoisomerase I (YpTOP) under the control of the BAD promoter was cloned from pYTOP into the MCS of pBRINT-T_sCM to construct the plasmid pBRYTOP. Site-directed mutagenesis of YpTOP at Asp117 to Asn or at Gly122 to Ser was carried out with the pBRYTOP as template. The integration plasmids (pBRYTOP or its mutant derivatives) were introduced into the *lacZ* region of the chromosome of *E. coli* (strain MG1655) by electroporation and transformants were selected after 24-48 h of incubation at 30°C. The resulting transformants were carbenicillin (Cb) and chloramphenicol (Cm) resistant. A single transformant colony was grown in 0.5-ml of LB medium for 4 h at 30°C. The resulting culture was then inoculated into 10-ml of LB medium and incubated overnight at 44°C. Several dilutions (10⁻³ - 10⁻⁶) of this overnight cultures were plated on LB agar supplemented with XGal, IPTG and Cm, and incubated at 44°C for 24 h. Single white colonies from these plates were screened for resistance to Cm (Cm^R) and sensitivity to Cb (Cb^S), at 44°C. White Cm^RCb^S colonies were replicated again at 44°C and checked for the Cb sensitivity at 30°C to confirm for the absence of the plasmid. The chromosomal integration was further verified by PCR. The integrated DNA was then moved to the desired strain BW27784 by P1 transduction followed by PCR verification to create strains BWYTOP (with chromosomally integrated YpTOP gene) and BW117N (with chromosomally integrated YpTOP-D117N gene).

Cell killing assay

Cultures of the *E. coli* strains expressing the recombinant YpTOP under the control of the BAD promoter in LB medium with antibiotics for selection were induced with the indicated concentration of arabinose for the stated time at 37°C or 30°C if strains had temperature-sensitive pBRYTOP and its derivatives. Serial dilutions of the culture were then plated on LB

plates with antibiotics and 2% glucose. After overnight incubation, the number of colonies obtained from arabinose induced culture was divided by the number of colonies from the non-induced control culture to obtain the relative viability.

Relaxation activity Assay

Serial dilutions of the topoisomerase proteins were added to 20 μ l of reaction buffer containing 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml gelatin, 6 mM MgCl₂, and 0.5 μ g of CsCl gradient-purified negatively supercoiled plasmid DNA. After incubation at 37°C for 30 min, the DNA was analyzed by gel electrophoresis in 1% agarose gel as described previously⁷. The relaxation activity was compared by the amount of enzyme required to achieve similar extent of supercoil removal.

DNA cleavage and religation assay

DNA cleavage activity was assayed using the 5'-³²P labeled 59 base oligonucleotide substrate¹⁷ in 5 μ l of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and the indicated concentration of MgCl₂ at 37°C for 10 min. For measurement of DNA religation activity at either 0°C or 37°C, 1 M NaCl was added to the cleavage reaction to dissociate the enzyme from the religated DNA. Incubation was continued at either 0°C or 37°C before the reactions were terminated at the indicated time points by addition of equal volume of stop solution (79% formamide, 0.2M NaOH, 0.04% bromophenol blue) and analyzed by electrophoresis in a 15% sequencing gel as described²⁷. For the 13 base oligonucleotide substrate used for EcTOP67, analysis was carried out with a 20% sequencing gel. The oligonucleotide substrate and DNA cleavage products were visualized and quantitated with the PhosphorImager Storm 860.

Comparison of Mg²⁺ binding affinity by Intrinsic Tryptophan Fluorescence Measurement

Wild-type and D113N mutant *E. coli* topoisomerase I proteins were dialyzed into 20 mM potassium phosphate, pH 7.4, 20 mM KCl. Change in tryptophan fluorescence of the protein at 0.1 mg/ml concentration upon addition of MgCl₂ was monitored using the CARY Eclipse fluorescence spectrophotometer at room temperature (~25°C) with excitation at 295 nm, and spectral bandwidths of 5 and 10 nm respectively for excitation and emission. The data was analyzed by GraphPad Prism software.

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Figure 1. YpTOP-D117N, C737Y enzyme had no relaxation activity but enhanced DNA cleavage activity

(A) Serial dilutions of wild-type YpTOP and YpTOP-D117N,C737Y proteins were assayed for relaxation of negatively supercoiled plasmid DNA for 30 min at 37°C under standard reaction conditions in the presence of 6 mM MgCl₂. (B) 400 ng of wild-type YpTOP and YpTOP-D117N, C737Y proteins were assayed for formation of cleavage product from 5'-³²P-labeled 59 base oligonucleotide substrate in the presence of the indicated concentrations of MgCl₂.

Figure 2. Western blot analysis of expression of mutant YpTOP-D117N protein

The monoclonal antibodies against *E. coli* topoisomerase I recognized both wild-type EcTOP encoded on the chromosome and the recombinant YpTOP protein which has a slower mobility when intact because of the presence of fusion tags. Some of the recombinant YpTOP proteins were degraded into lower molecular weight forms. (A) Comparison of expression of YpTOP-G122S and YpTOP-D117N proteins from plasmid pBRYTOP-G122S and pBRYTOP-D117N in BW27784 at 4.5 h after arabinose addition at 30°C. (B) Induction of YpTOP-D117N from single copy chromosomal integration in BW117N by different concentrations of arabinose at 2.5 h after arabinose addition at 37°C.

Figure 3. Relaxation and DNA cleavage Activity of YpTOP-D119N and YpTOP-E121Q mutant enzymes

(A) Serial dilutions of wild-type YpTOP, YpTOP-D119N and YpTOP-E121Q proteins were assayed for relaxation of negatively supercoiled plasmid DNA for 30 min at 37°C under standard reaction conditions in the presence of 6 mM MgCl₂. (B) Time course of relaxation reaction with 100 ng of wild-type YpTOP and YpTOP-D119N proteins. (C) Oligonucleotide cleavage by wild-type YpTOP, YpTOP-D119N and YpTOP-E121Q proteins in the absence and presence (2 mM) of added MgCl₂.

Figure 4. Enhanced DNA cleavage complex formation by EcTOP67-D111N

(A) Product from cleavage of 0.5 pmoles of 5'-³²P labeled 59 base oligonucleotide by 400 ng of the wild-type or mutant TOP67 proteins with 0 or 2 mM MgCl₂ added. (B) Cleavage of 0.5 pmoles of 5'-³²P labeled 13 base oligonucleotide (AATGCGCTTTGGG) by 400 ng of the wild-type or mutant TOP67 proteins with the indicated concentration of MgCl₂ added. (C) Religation of the 13 base oligonucleotide substrate at 0°C. For wild-type TOP67, the cleavage reaction was carried out with no MgCl₂ added for 10 min, 5 mM MgCl₂ was then added along with 1 M NaCl to monitor religation. For mutant TOP67-D111N, cleavage reaction was carried out with 5 mM MgCl₂ before the addition of 1 M NaCl to measure the level of religated DNA.

Figure 5. Effect of the D113N mutation on EcTOP enzyme activity

(A) Relaxation of supercoiled DNA (B) Cleavage of 5'-³²P labeled 59 base oligonucleotide required the addition of MgCl₂. (C) Comparison of rate of DNA religation by wild-type EcTOP and EcTOP-D113N at 37°C. Cleavage complex was formed by 400 ng of enzyme and 0.5 pmole of 5'-³²P labeled 59 base oligonucleotide in the presence of 2 mM MgCl₂, 1 M NaCl was then added to dissociate the protein from the religated cleavage product. After separation by gel electrophoresis, the levels of substrate and cleavage product were quantitated by PhosphorImager for analysis.

Figure 6. Mg^{2+} binding by EcTOP-D113N

. Decrease in intrinsic tryptophan fluorescence was used to compare binding of two Mg^{2+} by wild-type EcTOP and EcTOP-D113N enzymes. The data is curved fitted to equation for two binding sites (shown as line) by GraphPad Prism software.

Figure 7. Positions of the proposed Mg^{2+} coordinating residues in the structure of a complex between EcTOP67 and single-stranded DNA

Active site region of the complex between EcTOP67 (with the H365R mutation) and the single-stranded oligonucleotide 5'-ACTTCGGGATG -3'²⁸ with the active site nucleophile Tyr319 and the acidic triad of Asp111, Asp113 and Glu115 shown.

Table 1

Effect of overexpression of recombinant topoisomerase I mutants from high copy number plasmid pYTOP on the viability of *E. coli* JD5

Recombinant topoisomerase	Relative viability ²
Wild-type YpTOP	0.17 ± 0.11
YpTOP-G122S	$5.2 \times 10^{-4} \pm 1.8 \times 10^{-5}$
YpTOP39 ¹	$1.7 \times 10^{-5} \pm 5.3 \times 10^{-6}$
YpTOP-D117N, C608G	$7.3 \times 10^{-3} \pm 1.8 \times 10^{-3}$
YpTOP-D117N, C668F	$6.7 \times 10^{-3} \pm 2.6 \times 10^{-3}$
YpTOP-D117N, C737Y	$1.1 \times 10^{-4} \pm 3.8 \times 10^{-5}$
YpTOP-D116N, W866L	$5.5 \times 10^{-5} \pm 6.8 \times 10^{-6}$
YpTOP-C737Y	0.25 ± 0.05
YpTOP-D119N	$2.1 \times 10^{-5} \pm 1.5 \times 10^{-5}$
YpTOP-E121Q	$6.7 \times 10^{-2} \pm 4.9 \times 10^{-3}$

¹YpTOP39 has D80N, G94S and D117N mutations.

²Relative viability from overexpression of the mutant YpTOP proteins was measured by the ratio of viable colonies obtained after induction of the BAD promoter controlling the expression of the recombinant YpTOP protein with 0.2% arabinose for 2 h in comparison with the non-induced culture. The results represent the average and standard deviation of at least three measurements.

Table 2

Effect of expression of mutant recombinant *Y. pestis* topoisomerase I in single or very low copies on *E. coli* viability

Strain	[Arabinose], percent	Relative viability ²
MG1655/pBRYTOP ¹	0.2	0.61 ± 0.22
MG1655/pBRYTOP-G122S	0.2	0.28 ± 0.05
MG1655/pBRYTOP-D117N	0.2	$2.8 \times 10^{-4} \pm 1.6 \times 10^{-4}$
BW27784/pBRYTOP	0.2	0.84 ± 0.16
BW27784/pBRYTOP-G122S	0.2	0.26 ± 0.19
BW27784/pBRYTOP-D117N	0.2	$4.5 \times 10^{-5} \pm 4.2 \times 10^{-5}$
MGYTOP	0.2	0.85±0.18
MG122S	0.2	0.97±0.07
MG117N	0.2	$5.6 \times 10^{-3} \pm 1.4 \times 10^{-3}$
BWYTOP	0.0002	1.1 ± 0.06
BWYTOP	0.002	0.92 ± 0.33
BWYTOP	0.02	0.91 ± 0.08
BWYTOP	0.2	0.94 ± 0.01
BW117N	0.0002	$5.2 \times 10^{-4} \pm 4.3 \times 10^{-4}$
BW117N	0.002	$2.1 \times 10^{-4} \pm 3.0 \times 10^{-4}$
BW117N	0.02	$2.4 \times 10^{-5} \pm 1.9 \times 10^{-5}$
BW117N	0.2	$5.3 \times 10^{-5} \pm 3.3 \times 10^{-5}$

¹ Experiments with strains carrying pBRYTOP (with a ts-replicon) and its derivatives were carried out at 30°C.

² Relative viability from expression of the mutant YpTOP proteins was measured by the ratio of viable colonies obtained after induction of the BAD promoter controlling the expression of the recombinant YpTOP protein with the indicated concentration of arabinose in comparison with the non-induced culture. Serial dilutions to determine viable counts were carried out 4.5 h after addition of arabinose for strains with pBRYTOP and its derivatives. For MG1655 derived strains with integrated YpTOP gene, and BWYTOP and BW117N, dilutions were carried out 2.5 h after arabinose addition at 37°C. The results represent the average and standard deviation of at least three measurements.