Bacterial Cell Killing Mediated by Topoisomerase I DNA Cleavage Activity*

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DNA topoisomerases are important clinical targets for antibacterial and anticancer therapy. At least one type IA DNA topoisomerase can be found in every bacterium, making it a logical target for antibacterial agents that can convert the enzyme into poison by trapping its covalent complex with DNA. However, it has not been possible to previously observe the consequence of having such a stabilized covalent complex of bacterial topoisomerase I in vivo. We isolated a mutant of recombinant Yersinia pestis topoisomerase I that forms a stabilized covalent complex with DNA by screening for the ability to induce the SOS response in Escherichia coli. Overexpression of this mutant topoisomerase I resulted in bacterial cell death. From sequence analysis and site-directed mutagenesis, it was determined that a single amino acid substitution in the TOPRIM domain changing a strictly conserved glycine residue to serine in either the Y. pestis or E. coli topoisomerase I can result in a mutant enzyme that has the SOS-inducing and cell-killing properties. Analysis of the purified mutant enzymes showed that they have no relaxation activity but retain the ability to cleave DNA and form a covalent complex. These results demonstrate that perturbation of the active site region of bacterial topoisomerase I can result in stabilization of the covalent intermediate, with the in vivo consequence of bacterial cell death. Small molecules that induce similar perturbation in the enzyme-DNA complex should be candidates as leads for novel antibacterial agents.

DNA topoisomerases are ubiquitous enzymes that are needed either for control of DNA supercoiling or for overcoming topological barriers during replication, transcription, recombination, or repair (reviewed in Refs. 1–3). Type IB and type II DNA topoisomerases are well utilized targets of many clinically important anticancer and antibacterial drugs (4–7). These drugs cause cell death by stabilizing the covalent intermediate formed between topoisomerase protein and cleaved DNA during the catalytic cycle of the enzyme. There is at least one type IA DNA topoisomerase present in every genome examined so far. It is likely to be essential for overcoming topological barriers requiring single-stranded DNA passage (2). It has been proposed that bacterial type IA DNA topoisomerases could be a useful therapeutic target if small molecules that stabilize the covalent intermediate of this class of topoisomerases can be identified (8). However, it has never been demonstrated that stabilization of the covalent intermediate formed between a type IA topoisomerase and the cleaved single DNA strand can lead to cell death. It therefore remains unclear whether bacterial topoisomerase I can indeed be the target of "poison" molecules that would be bactericidal.

In this study, a mutant bacterial topoisomerase I that forms a stabilized covalent intermediate with cleaved DNA was identified via an SOS induction screen (9) in Escherichia coli. Overexpression of this mutant topoisomerase in E. coli led to extensive cell killing. DNA sequence analysis and site-directed mutagenesis showed that a single amino acid substitution at a strictly conserved glycine residue in the enzyme can confer the SOS-inducing and cell-killing properties. The study was first carried out with recombinant Y. pestis topoisomerase I because of the relevance of this pathogen to biodefense. A homologous Gly to Ser mutation in E. coli topoisomerase I was also found to lead to SOS induction and cell killing. Direct isolation of this E. coli topoisomerase I mutant from the SOS screen would probably have been unlikely because two simultaneous nucleotide changes in the same codon would be required. Analysis of the purified mutant enzymes showed that although DNA relaxation activity was abolished, the mutant enzymes could cleave DNA upon the addition of Mg(II) and form the covalent protein-DNA intermediate complex. A similarly positioned glycine residue is also strictly conserved in type II DNA topoisomerases and is part of the TOPRIM motif found in many nucleotidyl transferases including type IA and type II topoisomerases, DnaG-type primases, nucleases of the P2 phage OLD family, RecR proteins, and ribonuclease M5 (10–12). These results validate bacterial type IA topoisomerase as a potential therapeutic target, while illuminating structural features in the active sites of topoisomerases that may be important for maintaining the equilibrium between the DNA cleavage and religation activities of these enzymes.

MATERIALS AND METHODS

Recombinant Protein Expression Plasmids—Oligonucleotide primers used in cloning were synthesized by SIGMA Genosys. The Yersinia pestis topoisomerase I DNA coding sequence was amplified using the primers 5′-ATGGTTAAAGCTCTCGAATAG-3′ and 5′-TTCCTTCGCCCAAGCCCC-3′ and strain KIM10(+) genomic DNA as template with FidelityTaq polymerase (from USB). The PCR product was cloned into pBAD/Thio to create the plasmid pYTOP using the pBAD/TOPO Thiofusion expression kit (Invitrogen). The resulting recombinant Y. pestis topoisomerase I protein (YTOP)2 has a thioredoxin fusion at the N terminus and His6 fusion at the C terminus. For expression of the E. coli topoisomerase I under the control of the BAD promoter, a PCR product was generated from strain MG1655 genomic DNA with the primers 5′-CATAATCATGATGGGTAAAGCTCTTGTCATG-3′ and 5′-GATAGACGCTCGAGGATCCGATTCTGTCTGATCG-3′ (with the BspHI restriction site that shares compatible cohesive ends

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The abbreviations used are: YTOP, Y. pestis topoisomerase I protein; ETOP, E. coli topoisomerase I protein; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
with DNA cleaved by Ncol) and 5′-TTTAGATCTATTTTTTCTCATTACCCC-3′ using Pfu ultra DNA polymerase (from Stratagene). The PCR product was digested with the restriction enzyme BspHI and ligated to plasmid pBAD/Thio that had been digested with the restriction enzymes Pmel and Ncol. The resulting pETOP vector expresses the recombinant E. coli topoisomerase I with no fusion tags.

**Mutagenesis**—Random mutagenesis of the Y. pestis topoisomerase I was carried out using the GeneMorph II EZClone domain mutagenesis kit from Stratagene. The Y. pestis topA gene fragment was amplified using primers 5′-ATGGTAAAGCTCTGTTAATAG-3′ and 5′-TTTCTTGGCTCAACCCC-3′ and the Mutazyme II DNA polymerase for 25 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 3.5 min. The purified PCR product was then used in the GeneMorph II EZClone reaction to regenerate the pETOP plasmid for expression of the mutated topoisomerase I under the control of the BAD promoter. The plasmid expressing the randomly mutagenized topoisomerase I protein was first amplified in E. coli XL10-Gold Ultracompetent cells (from Stratagene) in LB medium with 100 μg/ml ampicillin and 2% glucose. The mutagenized plasmid library was then used to transform E. coli JD5 (13) with the dinD::lacZ fusion. The transformants were first isolated on LB plates with ampicillin and 2% glucose. They were then replica plated onto LB plates with ampicillin and 0.0002 or 0.002% arabinose and 35 μg/ml X-gal indicator to clone the clones with SOS-inducing recombinant topoisomerase I mutants. Site-directed mutagenesis of individual residues on Y. pestis and E. coli DNA topoisomerase I was carried out using the QuikChange site-directed mutagenesis kit from Stratagene.

**Effect of Recombinant Topoisomerase Synthesis on Viability**—Initial experiments were carried out with E. coli JD5 cells. In this strain, sublethal concentrations of mutant topoisomerase I were expressed under the screening conditions (0.0002 and 0.002% arabinose), and saturating concentration of arabinose (0.2%) was used to observe the killing effect. In strain BW27784 (obtained from the Yale E. coli Genetic Stock Center), the arabinose transporter araE gene has been placed under the control of a constitutive promoter (14) so that relatively low concentrations of arabinose (0.002 and 0.00002%) could result in effective expression of the recombinant mutant topoisomerase and cell killing. E. coli JD5 or BW27784 expressing the recombinant topoisomerase was grown in LB medium with 150 μg/ml ampicillin to A600 = 0.4 before the addition of the indicated concentration of arabinose. After 2 h at 37 °C, serial dilution of the cultures was performed in LB, and the cultures were plated on LB plates with 100 μg/ml ampicillin and 2% glucose. The viable counts were recorded after overnight incubation at 37 °C.

**Protein Purification**—Recombinant wild-type and mutant Y. pestis topoisomerase I proteins with the His6 tag at the C terminus expressed in JD5 were purified using the Qiagen nickel-nitrioltriacetic acid affinity column. The eluted proteins were dialyzed into 0.1 M potassium phosphate, pH 7.5, 0.2 mM dithiothreitol, 0.2 mM EDTA, 50% glycerol. The E. coli G1165 mutant topoisomerase I has no histidine tag and was purified as described for the wild-type enzyme (15).

**Assay of Relaxation Activity**—Relaxation activity was assayed in a reaction volume of 20 μl with 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml gelatin, 6 mM MgCl2, and 0.5 μg of CsCl gradient-purified supercoiled plasmid DNA. After incubation at 37 °C for 30 min, the reaction was terminated and analyzed by agarose gel electrophoresis as described previously (16).

**Assay of DNA Cleavage Activity**—To assay the cleavage of supercoiled plasmid DNA, 0.5 μg of plasmid DNA was incubated with the enzyme in 10 μl of 40 mM Tris-HCl, pH 7.6, 0.1 mM NaCl, 0.1 mM EDTA, and the indicated amount of MgCl2 at 37 °C for 20 min. The reaction was stopped by the addition of SDS to 1% and analyzed by gel electrophoresis in agarose gel containing 0.5 μg/ml ethidium bromide.

The 39-base oligonucleotide 5′-GATTATGCAATCGCTCTTCTGGCAACCAAGAGAGCATAAC-3′ has a preferred topoisomerase I cleavage site CAAT ↓ GC for E. coli DNA topoisomerase I (17). It was labeled at the 5′-end with T4 polynucleotide kinase and [γ-32P]ATP. Cleavage by wild-type and mutant topoisomerase I was assayed in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and the indicated concentration of MgCl2. The cleavage products were analyzed as described previously (18).

**Visualization of the Covalent Topoisomerase-DNA Complex**—To observe the covalent topoisomerase-DNA complex directly, the 9-base oligonucleotide 5′-CAATGGCGCT-3′ with the same preferred cleavage site CAAT ↓ GC was labeled with terminal deoxynucleotidyl transferase and [α-32P]dATP at the 3′-end. It was incubated with the topoisomerase in 10 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and the indicated amount of MgCl2 for 10 min. After addition of 2X loading buffer for SDS-PAGE, the reaction was incubated at 37 °C for another 10 min before heating at 100 °C for 5 min for SDS-PAGE analysis. The covalent complex in the dried gel was visualized with the PhosphorImager Storm 760.

**DNA Religation Assay**—To test the ability of the enzyme to religate the DNA after DNA cleavage, 1 mM NaCl was added to the 5′-end-labeled oligonucleotide cleavage reactions containing 5 mM MgCl2 and incubated for up to 10 min before termination of the reaction by the addition of an equal volume of stop solution (79% formamide, 0.2 mM NaOH, 0.04% bromophenol blue) for analysis by gel electrophoresis (18).

**Intrinsic Tryptophan Fluorescence Measurements**—Fluorescence measurements were carried out with the CARY Eclipse fluorescence spectrophotometer with excitation at 295 nm at room temperature (~25 °C). The spectral bandwidths were 5 and 10 nm, respectively, for excitation and emission. The wild-type and G1165 mutant E. coli topoisomerase I proteins were present at 0.1 mg/ml in 20 mM potassium phosphate, pH 7.4, 20 mM KCl.

**RESULTS**

**Isolation of an SOS-inducing Topoisomerase I Mutant**—The Y. pestis topoisomerase I gene was cloned by PCR into pBAD/Thio vector for tight regulation of expression under the araC-PBAD system (19) in the construction of plasmid pYTOP. After random mutagenesis of the recombinant topoisomerase coding sequence, the mutated pYTOP DNA was transformed into E. coli strain JD5 with a chromosomal dinD::lacZ (9) fusion that produces β-galactosidase when the E. coli SOS response is activated by DNA damage. The transformants were first isolated on LB plates with ampicillin and 2% glucose to suppress the potentially lethal expression of the recombinant Y. pestis topoisomerase I mutants. The transformants were then replica plated onto LB plates with ampicillin, arabinose, and X-gal to screen for mutant topoisomerase clones that induce the SOS response of E. coli. A clone that had the most distinct blue color on the indicator plate was designated pYTOP128 and analyzed by sequencing of the entire coding region of Y. pestis topoisomerase I. Three amino acid substitutions were identified: Gly-122 to Ser, Met-326 to Val, and Ala-383 to Pro. Among these positions, Ala-383 is the least conserved among the type IA DNA topoisomerases, with the proline substitution actually seen in some type IA topoisomerase sequences (Fig. 1a). The other two substitutions are at functionally important regions of the enzyme. Gly-122 is strictly conserved (Fig. 1a) and immediately follows the acidic triad DXDXE that has been postulated to coordinate two Mg(II) ions required for the relaxation activity of type IA DNA topoisomerases (16, 20). It is also part
Effect of overexpression of recombinant wild-type or mutant topoisomerase I on the viability of E. coli JD5

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

| Recombinant topoisomerase | Induced/non-induced relative viability |
|---------------------------|---------------------------------------|
| Wild-type YTOP            | 0.17 ± 0.11                           |
| YTOP128                   | 3.5 × 10^{-4} ± 2.5 × 10^{-5}         |
| YTOP128-Y325A             | 0.18 ± 0.096                          |
| YTOP-G122S                | 5.2 × 10^{-4} ± 1.8 × 10^{-5}         |
| YTOP-M326V                | 0.11 ± 0.01                           |
| YTOP-A383P                | 0.20 ± 0.16                           |
| Wild-type ETOP            | 0.56 ± 0.025                          |
| ETOP-G116S                | 9.3 × 10^{-4} ± 2.7 × 10^{-4}         |
| ETOP-G116S/Y319A          | 0.43 ± 0.23                           |

of the TOPRIM motif (10–12) involved not only in the catalysis of type IA DNA topoisomerase but also important for the activity of type II DNA topoisomerases (21, 22) and other nucleotidyl transferases that share a common requirement of Mg(II) for catalytic activity (10–12). A strictly conserved glycine also immediately follows the acidic triad in diverse type II DNA topoisomerase sequences (Fig. 1b). Met-326 immediately follows the active site tyrosine responsible for DNA cleavage and religation. It is not as strictly conserved as Gly-122, with a proline sometimes found in that position instead of methionine. The three amino acid substitutions were introduced individually into pYTOP by site-directed mutagenesis. Of the three resulting mutants, only the G122S mutant induced the SOS response when expressed in JD5. The active site tyrosine Y325A mutation was introduced by site-directed mutagenesis into wild-type pYTOP, pYTOP128, and pYTOP-G122S. The resulting active site mutants did not induce the SOS response of JD5, indicating that the SOS induction observed with pYTOP128 and pYTOP-G122S was not due to loss of relaxation activity but required the DNA cleavage function of the enzyme. The corresponding Gly-116 of E. coli DNA topoisomerase I, expressed from the BAD promoter on pETOP, was also mutated to Ser. The resulting ETOP-G116S mutant topoisomerase was found to similarly induce the SOS response when expressed in JD5 and plated on X-gal indicator plate with 0.0002% arabinose.

Cell Killing from Overexpression of the SOS-inducing Mutant Topoisomerases—The effect of the overexpression of the mutant topoisomerases on E. coli JD5 viability was examined by measuring the number of colonies obtained from cultures induced with 0.2% arabinose for 2 h in comparison with uninduced cultures to arrive at a viability ratio. The expression of YTOP128, YTOP-G122S, and ETOP-G116S resulted in extensive loss of viability (>1,000-fold) (TABLE ONE). Expression of the wild-type or the other mutant topoisomerases had only a minor effect on viability (<10-fold). The effect of the conserved Gly to Ser mutation on viability was again abolished when the active site tyrosine was mutated to alanine. Expression of the wild-type and mutant Y. pestis topoisomerase I had greater effects on viability than E. coli topoisomerase I probably because the recombinant Y. pestis topoisomerase I protein has an N-terminal thioredoxin fusion and was expressed at a higher level than recombinant E. coli topoisomerase I without the fusion tag (data not shown). A saturating concentration of arabinose (0.2%) was used in these experiments with JD5 to measure the effect on cell viability because it has been reported previously (23) that gene expression from plasmids containing the P_{BAD} promoter represents mixed populations with different quantities of the recombinant proteins because the araC-P_{BAD} system and the associated l-arabinose transporter AraE are regulated autocatalytically in the all-or-none manner. This drawback for utilizing the P_{BAD} system for gene regulation has been overcome by placing the low affinity high capacity AraE transporter under the control of constitutive promoters in the host strains constructed for uniform regulation of gene expression by the P_{BAD} Promoter (14). The synthesis of AraE becomes arabinose-independent, and the degree of induction is

![Figure 1. Positions of mutations found in pYTOP128 and their degree of conservation.](image-url)
Bacterial Cell Killing by Topoisomerase I DNA Cleavage

The SOS-inducing Mutant Topoisomerases Had No Relaxation Activity—The wild-type and mutant forms of the recombinant \( Y. \) \( \text{pestis} \) topoisomerase I were expressed and purified by nickel-nitrioltriacetic acid affinity column via the binding of the His tag present at the C terminus. The G116S mutant of \( E. \) \( \text{coli} \) topoisomerase I did not have the thioetherox or His tag attached. It was purified to homogeneity with the combination of chromatography columns used for wild-type \( E. \) \( \text{coli} \) DNA topoisomerase I. The relaxation and DNA cleavage activity of these purified topoisomerases were assayed with supercoiled plasmid DNA. The wild-type YTOP with the fusion tags had robust relaxation and cleavage activities (data not shown). In contrast, the YTOP-G122S and ETOP-G116S mutant topoisomerases had >400-fold loss of relaxation activity (Fig. 2).

The SOS-inducing Mutant Topoisomerases Could Cleave DNA and Form the Covalent Intermediate—Addition of Mg(II) was not required to observe cleavage of supercoiled plasmid DNA (Fig. 3, A and B) or a 5′-end-labeled single-stranded oligonucleotide (Fig. 3C) by wild-type \( E. \) \( \text{coli} \) or \( Y. \) \( \text{pestis} \) topoisomerase I, as reported previously for \( E. \) \( \text{coli} \) topoisomerase I (24). Although DNA cleavage could be observed for the Gly to Ser mutants, the DNA cleavage activities were found to be dependent on the addition of Mg(II) to the reaction mixture (Fig. 3). When the DNA cleavage activity was assayed with a short single-stranded oligonucleotide labeled with \(^{32}\)P at the 3′-end, the covalent complex formed by topoisomerase I could be observed by phosphorimaging after SDS gel electrophoresis (Fig. 4A). Formation of such a covalent complex by the ETOP-G116S mutant was confirmed to be Mg(II)-dependent (Fig. 4A). The covalent complex of ETOP1 formed with 5′-end-labeled oligonucleotide is religated at least partially in the presence of Mg(II), and the addition of high salt dissociates the enzyme from the DNA after religation (24, 25). This decrease of the level of cleaved DNA observed (>80%) because of DNA religation and dissociation by high salt was very rapid for wild-type \( E. \) \( \text{coli} \) DNA topoisomerase I (Fig. 4B). However, there was no decrease in the amount of cleaved DNA from religation observed for the G116S mutant after the addition of high salt (Fig. 4B).

Effect of arabinose concentration on the viability of \( E. \) \( \text{coli} \) BW27784 expressing recombinant wild-type ETOP or ETOP-G116S under the control of the BAD promoter

| Arabinose concentration | Relative viability ETOP | Relative viability ETOP-G116S |
|------------------------|------------------------|-----------------------------|
| %                      |                        |                             |
| 0                      | 2.2 ± 0.2              | 0.35 ± 0.03                 |
| 0.00002                | 0.86 ± 0.05            | 3.9 × 10^{-5} ± 1.1 × 10^{-3} |
| 0.0002                 | 0.66 ± 0.11            | 2.4 × 10^{-3} ± 3.6 × 10^{-5} |
| 0.002                  | 0.34 ± 0.05            | 7.1 × 10^{-5} ± 5.6 × 10^{-5} |

A. Wild-type YTOP
B. G122S-YTOP

FIGURE 2. Loss of relaxation activity from the mutation of the strictly conserved glycine to serine in \( Y. \) \( \text{pestis} \) and \( E. \) \( \text{coli} \) DNA topoisomerase I. Serial dilutions of wild-type and mutant (G122S-YP and G116S-ETOP) topoisomerase I proteins from \( Y. \) \( \text{pestis} \) (A) and \( E. \) \( \text{coli} \) (B) were assayed for relaxation activity with negatively supercoiled plasmid DNA.

The E. coli G116S Mutant Topoisomerase I Had Decreased Affinity for Mg(II)—It has been demonstrated previously that binding of Mg(II) to \( E. \) \( \text{coli} \) topoisomerase I results in a decrease in the maximum emission of tryptophan fluorescence of the enzyme (16, 26). The tryptophan fluorescence emission peak of ETOP-G116S was slightly below that of the wild-type enzyme (Fig. 5a). The tryptophan fluorescence emission of the ETOP-G116S mutant also decreased upon the addition of MgCl\(_2\). The maximal decrease in fluorescence observable for the G116S mutant was less than that observed for the wild-type enzyme (Fig. 5b). Around 0.7 mM Mg(II) was required to observe the maximal decrease of ETOP-G116S, compared with around 0.4 mM Mg(II) for the wild-type enzyme (Fig. 5c). Analysis of the data by non-linear regression showed better fit of the data to two sites binding versus one site binding, as expected from previous results from metal analysis that showed each wild-type enzyme binding to two Mg(II) ions (26). For wild-type ETOP, 0.112 and 67.6 \( \mu \text{M} \) were obtained as best fit values for \( K_{\text{d1}} \) and \( K_{\text{d2}} \) versus 1.39 and 235 \( \mu \text{M} \) for ETOP-G116S.

The Relaxation Activity of the E. coli G116S Mutant Topoisomerase I Could Not Be Restored by High Concentrations of Mg(II)—The relaxation activity of wild-type and G116S mutant \( E. \) \( \text{coli} \) topoisomerase I was assayed in a reaction mixture containing higher concentrations of MgCl\(_2\) compared with the 6 mM concentration in the standard assay conditions (Fig. 6). No relaxation activity could be observed for the G116S mutant topoisomerase I at MgCl\(_2\) concentrations as high as 26 mM.

DISCUSSION

Inside the bacterial cell, the covalent complex formed by wild-type topoisomerase I is an intermediate in between the DNA cleavage and religation steps during the relaxation cycle catalyzed by the enzyme. In contrast, these SOS-inducing topoisomerase I mutants identified in this study are expected to form a stabilized covalent complex with chromosomal DNA after the DNA cleavage step but cannot continue through the complete reaction cycle needed for the relaxation activity. This stabilized cleavage complex would then initiate the events that lead ultimately to cell death as measured by the data in TABLES ONE and TWO. Previous studies (27–29) with conditional lethal mutants of \( S. \) \( \text{aureus} \).
resulting from the substitution, as measured by the decrease in the tryptophan fluorescence signal upon the addition of Mg(II). This would explain the absence of DNA cleavage for ETOP-G116S and YTOP-G122S when MgCl₂ was not added to the cleavage reaction. However, this reduction in Mg(II) affinity is unlikely to account for the absence of recombination and relaxation activities from these mutants under reaction conditions with MgCl₂ present at greater than 5 mM. In previous studies (16), the Mg(II)-coordinating Asp-111, Asp-113, and Glu-115 residues in E. coli topoisomerase I were mutated to alanines in pairwise combinations. Although these pairwise substitutions resulted in significant decrease in Mg(II) binding affinity, the relaxation activity in all except the D111A/D113A combination was not lost completely and could be restored to wild-type level with 10–20 mM MgCl₂ present in the relaxation reaction (16). Expression of these acidic triad mutants did not induce the SOS response in JD5. The Gly to Ser mutation, in addition to affecting the Mg(II) binding affinity, is likely to also alter the active site structure inhibiting DNA recombination. Although DNA cleavage could take place with MgCl₂ added to the reaction mixture, relaxation activity was still not observed at MgCl₂ concentrations up to 26 mM. Gly-116 is near the N terminus of an α helix that protrudes into the active site pocket of E. coli topoisomerase I (Fig. 7). The G116S substitution is likely to influence Mg(II) coordination by the acidic triad. The substitution could also affect the positioning of the 3’-hydroxyl group formed from DNA cleavage by Tyr-319. The alteration of the relative positioning of the 3’-hydroxyl group and the phosphotyrosine linkage would have an effect on DNA religation and could account for the stabilization of the covalent cleaved complex and loss of relaxation activity. We could not detect significant differences in the GluC protease digestion patterns of the wild-type and G116S mutant ETOP enzymes (data not shown), so x-ray crystallography may be needed to reveal the perturbation in the active site resulting from the Gly to Ser substitution.

Drugs that stabilize the covalent cleaved complex formed by type IB toposomerase and Salmonella typhimurium gyrase showed that it could be the stabilization of the covalent cleaved complex and not necessarily the increase in the level of complex formed that might be responsible for the cell-killing effect of these mutants. The use of strain JD5 in our screening allowed the mutant to be identified at a sublethal concentration of arabinose. In strain BW27784, where efficiency of arabinose transport is not dependent on arabinose concentration, cell killing could be achieved at a very low concentration of arabinose.

The mutation to serine of the conserved glycine found in the TOPRIM motif DXDXG (10–12) was found to be sufficient to confer the SOS-inducing and cell-killing properties to Y. pestis and E. coli toposomerase I. Mutation of the glycine in the DXDXG motif of RNase M5 to alanine has been found to result in >30-fold loss of activity (12). The enzymes that have the TOPRIM motif all require Mg(II) for catalytic activity. Gly-116 of E. coli toposomerase immediately follows the acidic DXDXE triad shown previously to be involved in Mg(II) binding (16). Although the addition of Mg(II) is not required to observe DNA cleavage by wild-type Y. pestis and E. coli toposomerase I, it is possible that these enzymes retained bound Mg(II) during enzyme purification. The ETOP-G116S mutant had reduced affinity for Mg(II) resulting from the substitution, as measured by the decrease in the tryptophan fluorescence signal upon the addition of Mg(II). This would explain the absence of DNA cleavage for ETOP-G116S and YTOP-G122S when MgCl₂ was not added to the cleavage reaction. However, this reduction in Mg(II) affinity is unlikely to account for the absence of recombination and relaxation activities from these mutants under reaction conditions with MgCl₂ present at greater than 5 mM. In previous studies (16), the Mg(II)-coordinating Asp-111, Asp-113, and Glu-115 residues in E. coli topoisomerase I were mutated to alanines in pairwise combinations. Although these pairwise substitutions resulted in significant decrease in Mg(II) binding affinity, the relaxation activity in all except the D111A/D113A combination was not lost completely and could be restored to wild-type level with 10–20 mM MgCl₂ present in the relaxation reaction (16). Expression of these acidic triad mutants did not induce the SOS response in JD5. The Gly to Ser mutation, in addition to affecting the Mg(II) binding affinity, is likely to also alter the active site structure inhibiting DNA recombination. Although DNA cleavage could take place with MgCl₂ added to the reaction mixture, relaxation activity was still not observed at MgCl₂ concentrations up to 26 mM. Gly-116 is near the N terminus of an α helix that protrudes into the active site pocket of E. coli topoisomerase I (Fig. 7). The G116S substitution is likely to influence Mg(II) coordination by the acidic triad. The substitution could also affect the positioning of the 3’-hydroxyl group formed from DNA cleavage by Tyr-319. The alteration of the relative positioning of the 3’-hydroxyl group and the phosphotyrosine linkage would have an effect on DNA religation and could account for the stabilization of the covalent cleaved complex and loss of relaxation activity. We could not detect significant differences in the GluC protease digestion patterns of the wild-type and G116S mutant ETOP enzymes (data not shown), so x-ray crystallography may be needed to reveal the perturbation in the active site resulting from the Gly to Ser substitution.

Drugs that stabilize the covalent cleaved complex formed by type IB...
and type II DNA topoisomerases are widely applied for antibacterial and anticancer therapy (4–7). Although it has been postulated that bacterial type IA topoisomerases can be similarly utilized as a therapeutic target (8), this is the first demonstration that stabilization of the covalent cleaved complex formed by this class of topoisomerases can lead to bacterial cell killing. Small molecules that have the same effect as the conserved Gly to Ser mutation upon binding to the topoisomerase I active site could be promising leads for novel antibacterial agents. It could be argued that drug resistance to such antibacterial agents might develop from loss of topoisomerase I expression because the topA gene is not essential for viability of E. coli and related bacteria (30–32). However, the E. coli topA mutant is not viable in the absence of topoisomerase III function encoded by topB, so at least one type IA topoisomerase activity is required for viability (33). The Salmonella and Shigella topA mutants are viable (30, 31) probably only because topoisomerase III is still present. It should be possible to develop drugs that target both topoisomerase I and III, as there are fluoroquinolones that are active against both DNA gyrase and topoisomerase IV (34). Moreover, although the E. coli topA mutants are viable under standard laboratory growth conditions, they have greatly reduced survival rates when challenged by high temperature and oxidative stress (35, 36). During infection, these challenge conditions are part of the host defense, so potential resistance against topoisomerase I-targeting drugs through loss of topA gene expression would at the same time reduce the ability of the drug-resistant bacteria to survive the host challenge. Loss of the topA gene has also been shown more recently to decrease acid resistance of E. coli significantly (37). The recent emergence of multidrug-resistant bacterial pathogens in both community and hospital settings (38–40) represents an urgent public health problem. There is clearly a need to develop new antibacterial agents against a new target. Small molecules that stabilize the cleavage complex formed by bacterial type IA topoisomerase could provide useful leads for drug discovery.

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