The Strictly Conserved Arg-321 Residue in the Active Site of Escherichia coli Topoisomerase I Plays a Critical Role in DNA Rejoining*

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The strictly conserved arginine residue proximal to the active site tyrosine of type IA topoisomerases is required for the relaxation of supercoiled DNA and was hypothesized to be required for positioning of the scissile phosphate for DNA cleavage to take place. Mutants of recombinant Yersinia pestis topoisomerase I with hydrophobic substitutions at this position were found in genetic screening to exhibit a dominant lethal phenotype, resulting in drastic loss in Escherichia coli viability when overexpressed. In depth biochemical analysis of E. coli topoisomerase I with the corresponding Arg-321 mutation showed that DNA cleavage can still take place in the absence of this arginine function if Mg\(^{2+}\) is present to enhance the interaction of the enzyme with the scissile phosphate. However, DNA rejoining is inhibited in the absence of this conserved arginine, resulting in accumulation of the cleaved covalent intermediate and loss of relaxation activity. These new experimental results demonstrate that catalysis of DNA rejoining by type IA topoisomerases has a more stringent requirement than DNA cleavage. In addition to the divalent metal ions, the side chain of this arginine residue is required for the precise positioning of the phosphotyrosine linkage for nucleophilic attack by the 3’-OH end to result in DNA rejoining. Small molecules that can interfere or distort the enzyme-DNA interactions required for DNA rejoining by bacterial type IA topoisomerases could be developed into novel antibacterial drugs.

Cellular processes such as replication and transcription require strand separation of duplex DNA, which can potentially lead to excess DNA supercoiling or strand entanglement. DNA topoisomerases are enzymes that overcome the topological barriers in DNA for cellular processes to proceed at optimal rates (1). These enzymes function by transiently cleaving and rejoining DNA through the formation of an intermediate covalent enzyme-DNA complex (1, 2). Topoisomerases are divided into two types based on the number of strands they cleave. Type I topoisomerases cleave a single strand of DNA, whereas type II topoisomerases cleave both strands of DNA. Each type is further subdivided into subgroups that are functionally and structurally dissimilar (1).

Type IB and type IIA topoisomerases are well utilized targets for various anticancer and antibacterial drugs used in therapy. These drugs are effective in killing cancer and bacterial cells because they lead to the accumulation of the covalent intermediate formed between topoisomerase and cleaved DNA (3–7). The emergence of bacterial pathogens resistant to all available antibiotics poses a serious global public health problem. Hence, there is an urgent need for the discovery of a new class of antibacterial compounds. The type IA topoisomerase family includes bacterial and archael topoisomerase I, topoisomerase III, and eukaryotic topoisomerase III (8, 9). At least one type IA topoisomerase is present in each bacterial genome sequenced and annotated thus far (10, 11). Hence, type IA topoisomerases in bacteria represent a potential target for the discovery of new antibiotics. However, to identify potential interaction sites for the drugs, a detailed understanding of the biochemical interactions affecting DNA cleavage-religation in the active site of type IA topoisomerases is needed.

All DNA topoisomerases utilize an active site tyrosine hydroxyl nucleophile that attacks DNA phosphodiester linkage and forms the covalent intermediate with the cleaved DNA (12, 13). Quinolone drugs targeting type IIA bacterial topoisomerases act by trapping the enzyme on the cleaved DNA as a covalent cleavage complex, leading to bacterial cell death (6). Previous studies have demonstrated that the action of quinolones on DNA gyrase lead to the induction of the SOS response, a bacterial DNA repair response, in E. coli (14) due to the accumulation of the cleaved DNA complex.

The crystal structure of the 67-kDa N-terminal fragment of E. coli topoisomerase I revealed the presence of a number of acidic and basic residues in the active site region (15). Two basic residues, Lys-13 and Arg-321 are required for the relaxation activities and have been proposed to interact with the DNA phosphates for DNA cleavage (16–19). Three acidic residues Asp-111, Asp-113, and Glu-115 have been proposed to be part of the TOPRIM motif DDXDXG conserved in nucleotidyl transferases for binding of divalent ions required for catalytic activity (20). To identify mutations in bacterial topoisomerase I that can result in accumulation of DNA cleavage product, screening of SOS inducing mutations in recombinant Yersinia pestis topoisomerase I was carried out. These potentially dominant lethal...
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Identification of Y. pestis Topoisomerase I Mutants with Deficiency in DNA Rejoining—E. coli strain JD5 containing the chromosomal dinD1::lacZ fusion as SOS response reporter was transformed with plasmid pYPOT expressing Y. pestis topoisomerase I (YP TOP1) under the control of the BAD promoter (22). Random mutations were introduced into the YpTOP1 coding sequence by PCR (22). The transformants were first isolated on Luria-Bertani (LB) plates with 100 μg/ml ampicillin. Mutant clones that induce the SOS response were identified as blue colonies and maintained in LB with 2% glucose and 100 μg/ml ampicillin. Mutant clones were obtained from induced culture with the number of colonies obtained from the noninduced culture.

Expression and Purification of Wild Type E. coli Topoisomerase I and R321L, R321F, and R321K Mutants—The E. coli topoisomerase I (EcTOP1) R321L, R321F and R321K mutant clones were generated by site-directed mutagenesis using the QuikChange procedures with Pfui Ultra II Fusion HS DNA polymerase (Stratagene) and plasmid pLIC-ETOP as a template. The pLIC-ETOP plasmid has the EcTOP1 coding sequence expressed under the control of the T7 promoter with a tobacco etch virus protease cleavable N-terminal hexahistidine tag (26–28). Expression of the recombinant EcTOP1 proteins (wild-type and mutants) was induced in E. coli strain BL21AI (Invitrogen), which has the T7 RNA polymerase coding sequence under the control of lacI and the BAD promoter, by the addition of 0.02% arabinose and 1 mM isopropyl-β-D-galactopyranoside to culture when A600 = 0.4. Cells were collected after 3 h at 37 °C and lyzed in lysis buffer (50 mM sodium phosphate, pH 8.0, 0.3 M NaCl, 10 mM imidazole) by lysozyme treatment and freeze-thaw cycles as described previously (24). The recombinant proteins in the soluble lysate were allowed to bind to Ni-NTA-agarose (Qiagen) in wash buffer (50 mM sodium phosphate, 0.3 M NaCl, 20 mM imidazole, pH 8.0) by mixing, and the Ni-NTA-agarose was then packed into a column. After extensive washing, the proteins were eluted with elution buffer (50 mM sodium phosphate, 0.3 M NaCl, 250 mM imidazole, pH 8.0). The recombinant proteins were then cleaved with tobacco etch virus protease and passed through a Ni-NTA-agarose column again to remove the hexahistidine tag. Final purification was carried out with a single-stranded DNA cellulose column (Sigma) using increasing concentration of KCl gradient for elution of protein to achieve >99% homogeneity as observed in a Coomassie-stained SDS gel.

Assay of Relaxation Activity—Wild-type EcTOP1 and the Arg-321 substitution mutants were serially diluted and assayed for relaxation activity in a reaction volume of 20 μl. The reaction mixture contained 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml gelatin, 5 mM MgCl2, and 0.2 μg of supercoiled pBAD/thio plasmid DNA unless otherwise indicated. After incubation at 37°C for 30 min, the reaction was stopped by adding 4 μl of stop solution (50 mM EDTA, 50% glycerol, and 0.5% v/v bromphenol blue). The DNA was electrophoresed in a

Mutations in recombinant Y. pestis topoisomerase I can be more stably maintained in E. coli than the corresponding E. coli topoisomerase I mutants. Mutation of the glycine in the TOPRIM motif to serine was first identified for inhibition of DNA religation and enhanced cell killing due to accumulation of the covalent cleavage complex (22). The substitution of the first aspartate residue in the TOPRIM motif with asparagine has been shown to be an even more lethal mutation (23), demonstrating the requirement of Mg2+ for DNA religation even though Mg2+ is not absolutely required for DNA cleavage. In an attempt to determine what other biochemical perturbations can inhibit DNA rejoining, screening was carried out to identify additional random mutations of Y. pestis topoisomerase I that can result in SOS induction. It was observed that substitution of the strictly conserved arginine residue proximal to the active site tyrosine with serine led to induction of SOS response and bacterial cell death. Site-directed mutagenesis showed that mutating this arginine residue (Arg-327 in Y. pestis topoisomerase I) to a hydrophobic residue led to the maximum level of SOS induction and cell killing. The alanine substitution mutant of the corresponding Arg-321 in E. coli topoisomerase I has been studied previously and shown to be defective in DNA cleavage and relaxation of supercoiled DNA (17, 18, 24). Based on the crystal structure of E. coli topoisomerase III bound to single-stranded DNA, it was proposed that interaction of this strictly conserved Lys residue is required for interaction with the scissile phosphate in DNA cleavage (19, 25). To understand the biochemical basis for decreased cell viability observed for the Arg-327 mutants of Y. pestis topoisomerase I, Arg-321 of E. coli topoisomerase I was mutated to phenylalanine and leucine by site-directed mutagenesis. An additional mutation to a lysine residue was also made to study the effect of retaining the positive charge on the side chain. These E. coli topoisomerase I mutants were expressed, purified, and characterized in biochemical analysis of the partial steps of the relaxation cycle. The results showed that the topoisomerase I mutant lacking the Arg-321 function can carry out DNA cleavage if Mg2+ is present. However, the rate of DNA rejoining is greatly reduced when this conserved Arg residue is substituted with a hydrophobic residue. The requirement of specific biochemical interactions is thus significantly less stringent for catalysis of DNA cleavage compared with DNA rejoining. Positive charges from both Mg2+ ions and this Arg residue must be present for the 3'–OH end of the cleaved DNA to attack the phosphotyrosine linkage and rejoin the DNA backbone.

**EXPERIMENTAL PROCEDURES**

Identification of Y. pestis Topoisomerase I Mutants with Deficiency in DNA Rejoining—E. coli strain JD5 containing the chromosomal dinD1::lacZ fusion as SOS response reporter was transformed with plasmid pYPOT expressing Y. pestis topoisomerase I (YP TOP1) under the control of the BAD promoter (22). Random mutations were introduced into the YpTOP1 coding sequence by PCR (22). The transformants were first isolated on Luria-Bertani (LB) plates with 100 μg/ml ampicillin.

2 The abbreviations used are: YpTOP1, Y. pestis topoisomerase I; Ni-NTA, nickel-nitrilotriacetic acid; EcTOP1, E. coli topoisomerase I.
0.8% agarose gel, stained with ethidium bromide, and photographed over UV light.

**Assay of DNA Cleavage and Religation Activity**—For all cleavage and religation assays, a 59-base oligonucleotide (5’-GCCCTGAAGATTATCGATGCCTTTGGCCAAAA-CGAAGAGCATAATCTTTGAGGC-3’), with cleavage site CGCT ↓ TTTG, labeled with [γ-32P]ATP at the 5’ end, was used. For the cleavage assay, wild-type and mutant topoisomerases were incubated in the presence or absence of 0.5 mM MgCl₂ with the 32P-labeled oligonucleotide substrate at 37 °C for 10 min. For assaying the effect of subsequent addition of EDTA, the reactions were incubated with and without 5 mM EDTA being added at 37 °C for an additional 10 min. Following incubation, the reaction was stopped by adding equal volume of stop solution (79% formamide, 0.2 M NaOH, 0.04% bromphenol blue). The samples were heated at 95 °C for 5 min before electrophoresis in a 15% sequencing gel. The percentage of oligonucleotide cleaved by the enzymes was determined by analysis with the PhosphorImager Storm 860.

For wild-type EcTOP1 topoisomerase religation assay, the enzyme-oligonucleotide cleavage reaction with no MgCl₂ or EDTA present was carried out at 37 °C. The reaction mixture was then cooled on ice for 10 min post-incubation. To initiate religation, 0.5 mM MgCl₂ were added along with high salt (1 M NaCl) to dissociate the enzyme from DNA following the religation reaction at 0 °C on ice. For the mutant topoisomerases, the cleavage reaction mixture included 0.5 mM MgCl₂ and 1 mM NaCl was added to dissociate the enzyme from the DNA substrate following religation. All reactions were stopped at different time points by adding equal volume of stop solution. The samples were heated at 95 °C for 5 min before electrophoresis in a 15% sequencing gel. The percentage of oligonucleotide rejoined by the enzymes was determined by the disappearance of the cleaved product quantitated after analysis with the PhosphorImager Storm 860.

**Oligonucleotide Binding Assayed with Anisotropy Measurements**—The Varian Cary Eclipse fluorescence spectrophotometer was used for anisotropy measurements. Excitation wavelength was 495 nm, and emission wavelength was 520 nm. The excitation and emission slits were set at 5 and 10 nm, respectively. To measure binding, increasing concentrations (2–210 nM) of wild-type or mutant topoisomerase I were titrated into 5, 10, 15, 20, 25, and 30 nM solutions of oligonucleotide substrate used in the cleavage-religation assay modified with 6-carboxyfluorescein at the 3’ end (synthesized by Biosearch Technologies) in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA) with 1 mM MgCl₂ when indicated (29). The measurements were carried out at room temperature. Control experiments were performed by titrating the labeled oligonucleotide with volume of buffer corresponding to the enzyme additions. The increase in volume from addition of buffer or enzyme did not exceed 3.5% of the initial volume. Binding data were fit with the GraphPad Prism program according to Equation 1 for binding ligand depletion using anisotropy.

\[ Y = (A_{\text{max}}/z) \times \left( b - \sqrt{b \times b - 4 \times a \times c}\right)/(2 \times a) \]  
\[(\text{Eq. } 1)\]

In this equation, \(a = 1, b = K_d + X/n + z, c = X/n \times z, \) and \(K_d\) is the dissociation constant, \(n\) is the number of titrant molecules/fluorescent molecule, \(z\) is the fluorescent substance concentration, and \(A_{\text{max}}\) is the maximum relative anisotropy value.

### RESULTS

**Isolation of a Novel Topoisomerase I Mutant That Induces SOS Response**—The coding sequence of \(Y.\) pestis topoisomerase I gene under the control of the BAD promoter in plasmid pYTOP was subjected to random mutagenesis (22). The mutated pYTOP DNA was then transformed into \(E.\) coli strain JD5, which has a chromosomal dinD1::lacZ fusion that produces β-galactosidase when the SOS response is activated due to DNA damage (30). The transformants were initially isolated in LB plates with ampicillin and 2% glucose followed by replica plating onto LB plates with ampicillin, arabinose, and X-Gal to screen for SOS inducing clones. One of the clones that displayed a deep blue color on the X-Gal indicator plate was designated mutant AW3-75. Three amino acid substitutions were identified by sequence analysis of this clone: Thr-265 to Met, Arg-227 to Ser and Val-559 to Ile. Among these amino acids, Val-559 (corresponding to Val-555 in EcTOP1) is conserved as a strictly conserved residue in type IA topoisomerases (31). Thr-265 (corresponding to Ser-259 in EcTOP1) is not conserved in type IA topoisomerases, and many different substitutions can be found (31). Arg-327 (corresponding to Arg-321 in EcTOP1), which is located proximal to the active site tyrosine residue Tyr-325 in subdomain III, is a strictly conserved residue in type IA topoisomerases. The three amino acid substitutions were then individually introduced into pYTOP by site directed mutagenesis. Arabinose induction of the three resulting mutants in JD5 cells showed that only R327S led to the SOS response and had a cell killing effect when overexpressed (Table 1).

| Genotype | Induced/noninduced viability ratio |
|----------|-----------------------------------|
| Wild-type | 0.17 ± 0.11                       |
| AW3-75   | 7.8 × 10⁻⁵ ± 2.3 × 10⁻⁵           |
| R327K    | 2.5 × 10⁻⁵ ± 1.4 × 10⁻⁵           |
| R327A    | 1.1 × 10⁻⁴ ± 2.5 × 10⁻⁵           |
| R327S    | 2.7 × 10⁻⁴ ± 1.3 × 10⁻⁴           |
| R327L    | 3.2 × 10⁻⁴ ± 5.5 × 10⁻⁵           |
| R327W    | 7.7 × 10⁻⁴ ± 2.5 × 10⁻⁵           |
| R327T    | 8.3 × 10⁻⁴ ± 9.1 × 10⁻⁵           |
| R327F    | 6.7 × 10⁻⁵ ± 1.7 × 10⁻⁵           |

Based on this observation, we screened all possible amino acid substitutions at Arg-327 created by site-directed mutagenesis for SOS induction and found that all the mutants could induce the SOS response from the dinD1::lacZ reporter in JD5 cells to some extent when overexpression was induced by 0.002% arabinose as indicated by the blue color of the colonies on X-Gal indicator plate. This is in contrast with the results observed previously for substitutions at the conserved glycine in the TOPRIM domain where none of the substitution mutations except glycine to serine could induce the SOS response (32). Substitution of this conserved arginine in the active site with the hydrophobic residues Ile, Leu, Phe, and Trp had the strongest effects as indicated by the strong color intensity and
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FIGURE 1. Growth of E. coli AS17 transformed with pLIC-ETOP or the R321K, R321L, and R321F mutant derivatives at 30 and 42 °C. Serial 10-fold dilutions of cultures grown at 30 °C were spotted on LB plates and incubated overnight at 42 °C for 18 h or at 30 °C for 48 h.

The LIC-ETOP expression plasmids were transformed into E. coli BL21AI strain to induce expression from the T7 promoter to a high level for purification. In BL21AI, the transcription of the T7 RNA polymerase gene is under the control of the BAD promoter, and induction of recombinant topoisomerase I expression was achieved by the addition of 0.02% arabinose. Significant loss of viability (measured by viable colony counts from induced versus noninduced cultures after spreading on LB plates and incubation overnight) after 3 h of induction was observed for the cultures expressing the R321L (620-fold less colonies from induced culture versus noninduced culture) and R321F (329-fold) mutants but to a much lesser extent for cultures expressing the wild-type (2-fold) and R321K (6-fold) EcTOP1. This result is in agreement with the dominant lethal phenotype observed for the YpTOP1 hydrophobic substitution mutants at this conserved arginine.

FIGURE 2. Relaxation assay comparing wild-type EcTOP1 activity against the Arg-321 substitution mutants. Wild-type EcTOP1 and R321L, R321F, and R321K mutant enzymes (Enz) were serially diluted and added to a reaction mixture containing 0.2 μg of negatively supercoiled plasmid DNA. Incubation was at 37 °C for 30 min.

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Expression of Arg-321 Substitution Mutants of EcTOP1—The effect of substitution of this strictly conserved arginine on cell viability led us to hypothesize that the substitution mutations may result in accumulation of the topoisomerase cleavage complex in vivo. Previous studies with the alanine substitution mutant at the corresponding Arg-321 residue of E. coli topoisomerase I have suggested that this residue is required for DNA cleavage (17, 18), so the substitution mutants at the equivalent arginine in Y. pestis topoisomerase I were not expected to accumulate the cleavage complex. It would be a somewhat surprising result if the hydrophobic substitution mutants at this conserved arginine residue could still carry out DNA cleavage. To analyze the biochemical consequence of substituting this conserved arginine residue with a hydrophobic residue, E. coli topoisomerase I mutants with the hydrophobic substitutions of Phe, Trp, Leu, and Ile at Arg-321 were constructed for expression under the control of the T7 promoter in vector LIC-ETOP. However, only the R321L and R321F mutants could be expressed in sufficient levels for purification and biochemical analysis. In addition, the E. coli topoisomerase I R321K mutant was also characterized to determine the effect of retaining a positive charge at this position.

In a previous study (33), it was shown that background noninduced expression of bacterial topoisomerase I under the control of the T7 promoter in an expression plasmid can complement growth of E. coli strain AS17 with a temperature-sensitive chromosomal topA mutation (F’ topA17(arm) pLLI(Tet supD43,74)) (34) for growth at nonpermissive temperature. We found that although background expression of wild-type LIC-ETOP can complement growth of AS17 at 42 °C, AS17 cells transformed with plasmid carrying R321K, R321F, and R321L mutants had reduced viability at the nonpermissive temperature, suggesting deficiency in the relaxation activity (Fig. 1). The R321K mutant provided slightly better complementation for growth at the nonpermissive temperature when compared with the other mutants.

Relatively small size of the colonies on plates with X-Gal and 0.002% arabinose. Table 1 shows the consequence of induction of selected Arg-327 mutants on viability. Induction of the R327I, R327F, R327W, and R327L mutants with the high concentration of 0.2% arabinose resulted in >104-fold decrease in viable colony counts when compared with noninduced cells. The single R327S mutation identified in the original AW3-75 mutant had a lesser effect than all of the hydrophobic substitutions but did result in higher rate of cell killing than the alanine and lysine substitutions when the mutant topoisomerase I expression was induced with 0.2% arabinose.

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Loss of Relaxation Activities for Arg-321 Mutants of EcTOP1—The relaxation activity of the purified R321K, R321F, and R321L mutants was compared against wild-type E. coli topoisomerase I (EcTOP1) by incubation with negatively supercoiled plasmid DNA in buffer containing MgCl2. The results showed that the R321L and R321F mutations resulted in complete loss of relaxation activity (Fig. 2). The R321K mutant retained only ~3% of the relaxation activity. This is consistent with the in vivo complementation results observed with strain AS17. These results indicated that the hydrophobic substitution at Arg-321 led to severe disruption of the catalytic cycle of the enzyme as expected, and the lysine substitution that retained the positive charge was also not effective in providing the catalytic function of this conserved arginine residue.

Arg-321 Substitution Mutations Did Not Affect Binding Affinity of DNA Substrate—The catalytic cycle of bacterial topoisomerase I consists of the following steps: 1) binding of the enzyme to a single-stranded region of negatively supercoiled DNA; 2) cleavage of the single strand of DNA to allow for strand passage of the other strand of the duplex DNA; and 3) rejoining of the cleaved strand after strand passage has occurred. To measure binding affinity to the DNA substrate, a 59-base oligonucleotide, modified with 6-carboxyfluorescein at the 3’ end was used as the substrate. This oligonucleotide forms a hair pin structure and has a preferred cleavage site in the single-stranded region. Six different concentrations of the labeled oligonucleotide were titrated with increasing concentrations of either the purified wild-type, active site mutant Y319F (Fig. 3)
or the Arg-321 mutant enzymes (data not shown), and the change in anisotropy was measured. The data obtained were fit using an equation for binding ligand depletion using anisotropy, yielding the $K_d$ values. Table 2 shows that all the Arg-321 mutants examined here had $K_d$ values comparable with that of wild-type EcTOP1 indicating that the substitution of Arg-321 with a hydrophobic residue does not affect DNA binding affinity significantly. The active site Tyr-to-Phe mutation had little effect on the anisotropy results, probably because the cleavage complex constitutes only a very small fraction of the overall enzyme-DNA complex. By the same reasoning, omission of MgCl$_2$ did not affect the anisotropy results for the R321K mutant even though the absence of Mg$^{2+}$ prevents DNA cleavage by the R321K enzyme.

DNA Cleavage by Wild-type EcTOP1 and Arg-321 Substitution Mutants in Presence or Absence of Mg$^{2+}$ and EDTA—To determine whether the second step of the catalytic cycle, i.e. cleavage of DNA, is affected by the Arg-321 substitution mutations, the oligonucleotide substrate was labeled with $^32$P at the 5’ end. Wild-type EcTOP1 does not require Mg$^{2+}$ for DNA cleavage (Fig. 4, lane 2), and addition of 5 mM EDTA had no effect on the level of the DNA cleavage product (lane 3). However, the cleavage-religation equilibrium for wild-type EcTOP1 is shifted toward religation in the presence of 0.5 mM Mg$^{2+}$ as indicated by the disappearance of the cleavage product (lane 4), and the addition of excess EDTA (5 mM) over Mg$^{2+}$ (lane 5) reversed the equilibrium again toward DNA cleavage. In contrast, all three Arg-321 substitution mutants were incapable of DNA cleavage in the absence of Mg$^{2+}$ (lanes 6, 10, and 14). Addition of 0.5 mM Mg$^{2+}$ led to formation of DNA cleavage products by the mutants (lanes 8, 12, and 16). However, the level of cleaved product was lower than that observed for wild-type EcTOP1 in lane 2. Quantitation of results from three independent experiments by densitometry showed that the level of cleavage products in lanes 8, 12, and 16 for the R321L, R321F, and R321K mutant enzymes in the presence or absence of Mg$^{2+}$ was 42, 39, and 43%, respectively, of the cleavage product level from the wild-type enzyme in lane 1. Increasing the Mg$^{2+}$ concentration to 2 mM did not further increase the relative yield of the cleavage products from the mutants (data not shown). The cleavage products observed for R321L and R321F remained after 10 min of incubation following the addition of 5 mM EDTA (lanes 9 and 13), indicating that removal of the Mg$^{2+}$ did not reverse the DNA cleavage. The cleavage product observed for the R321K mutant formed in the presence of Mg$^{2+}$ decreased partially following the subsequent addition of 5 mM EDTA (lane 17).

DNA Religation by Wild-type EcTOP1 and Arg-321 Substitution Mutants—Experiments were next carried out to determine the effect of the mutations on the rate and extent of religation of DNA. As mentioned earlier, wild-type EcTOP1 does not require Mg$^{2+}$ for cleavage but requires the divalent ions for religation (35). Following addition of Mg$^{2+}$ post cleavage, rapid and complete religation of the cleaved product by wild-type EcTOP1 could be observed as disappearance of the cleavage product (Fig. 5A). In contrast, the rate of religation of the cleav-
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Effect of Arg-321 Mutation on Mg$^{2+}$ Concentration Requirement for Relaxation Reaction—The relaxation activity of the Arg-321 mutants have been analyzed under standard conditions in buffer containing 5 mM MgCl$_2$. Because these mutations resulted in a need for Mg$^{2+}$ in the DNA cleavage step not observed for wild-type bacterial topoisomerase I, the Mg$^{2+}$ concentration for requirement for maximal relaxation reaction catalyzed by the R321K EcTOP1 mutant was compared with that of the wild-type enzyme. A higher amount of R321K enzyme (400 ng at 200 nM final concentration) was used to observe comparable activity as 10 ng (5 nM final concentration) of the wild-type enzyme. The results (Fig. 6) showed that while relaxation activity of wild-type EcTOP1 reached a maximum at 2 mM MgCl$_2$, the relaxation activity of the R321K did not plateau until 4 mM MgCl$_2$. The R321L and R321F mutants were assayed at MgCl$_2$ concentrations of 6–25 mM (data not shown), with no observable relaxation activity even at these elevated MgCl$_2$ concentrations. The relaxation activity of these two mutants with hydrophobic substitutions at Arg-321 could not be rescued with high MgCl$_2$ concentrations.

**DISCUSSION**

The use of recombinant Y. pestis topoisomerase I with inducible expression in E. coli has been useful for identifying mutations in bacterial topoisomerase I that affect the DNA rejoining step. The Y. pestis topoisomerase I protein sequence shares 85% identity with E. coli topoisomerase I protein sequence, so mutations at analogous positions have been shown to have similar effects in both enzymes (22, 28, 32). Mutations affecting DNA rejoining are expected to be highly lethal due to accumulation of the cleaved covalent complex, and recombinant clones of such Y. pestis topoisomerase I mutants were found to be more stably maintained in E. coli than analogous recombinant E. coli topoisomerase I mutant clones. Characterization of a novel SOS-inducing Y. pestis topoisomerase I mutant isolated using the dinD1::lacZ reporter showed that substitution at the strictly conserved Arg proximal to the active site Tyr (corresponding to Arg-321 in E. coli topoisomerase I) can result in SOS induction and cell death when the mutants were overexpressed, suggesting a critical role of this residue in DNA rejoining. Previous studies showed that mutation of E. coli topoisomerase I Arg-321 to alanine resulted in a significant loss in the relaxation activity of the enzyme (17, 18). The crystal structure of E. coli topoisomerase III bound to an eight-base single-stranded oligonucleotide showed that the Arg-330 residue analogous to Arg-321 in Y. pestis topoisomerase I interacts with the scissile phosphate (25). Our results show that mutation of this arginine to a hydrophobic residue in recombinant Y. pestis topoisomerase I resulted in the most drastic cell killing among all the possible amino acid substitutions at this position. When the E. coli Arg-321 mutants were analyzed biochemically, it was observed that mutation of this residue did not affect the affinity of the enzyme for binding to DNA substrate significantly (Table 2). Further experiments demonstrated that mutation of Arg-321 in the active site of E. coli topoisomerase I led to a requirement of metal ion for cleavage (Fig. 4, lanes 8, 12, and 16). DNA cleavage
by these arginine substitution mutants would therefore take place under in vivo conditions in the presence of Mg\(^{2+}\). However, even in the presence of metal ions, these mutants were inefficient at religating DNA (Fig. 5). Mutation to a positively charged lysine residue could only replace the function partially, resulting in 3'-OH generated from DNA cleavage, metal ions are required for religation by the enzyme. Hence, it is proposed that the lack of this lysine residue in type II topoisomerases creates the requirement of an additional metal ion for cleavage of DNA (19). Based on previously published data and our new findings, it can be concluded that positioning of the DNA substrate for cleavage and religation by type IA topoisomerases involves divalent metal ion coordination and a network of amino acids including Arg-321 and Lys-13. The function of Arg-321 is dispensable for DNA cleavage if divalent ions are present but is critical for DNA religation even in the presence of divalent ions. The requirements for DNA religation are found to be more stringent than for DNA cleavage. This property of bacterial topoisomerase I may render it susceptible to a "poison" inhibitor that can inhibit DNA rejoining but has relatively insignificant effect on the DNA cleavage step, resulting in accumulation of the covalent intermediate formed with cleaved DNA.

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