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Hydroxyl Radicals Are Involved in Cell Killing by the Bacterial Topoisomerase I Cleavage Complex▽†

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Escherichia coli **expressing SOS-inducing mutant topoisomerase I was utilized to demonstrate that covalent protein-DNA complex accumulation results in oxidative damage. Hydroxyl radicals were detected following mutant topoisomerase induction. The presence of the Fe^{2+} chelator 2,2′-dipyridyl and an *iscS* mutation affecting Fe-S cluster formation protect against topoisomerase I cleavage complex-mediated cell killing.**

DNA topoisomerases are ubiquitous enzymes that carry out catalysis by coupling DNA strand passage with concerted breaking and rejoining of DNA (31). *Escherichia coli* DNA topoisomerase I, encoded by the *topA* gene, is the most studied member of the type IA topoisomerase family (40, 42). It is important for regulation of DNA supercoiling (41, 43) and has an essential function for preventing hypernegative supercoiling and R-loop formation during transcription (9, 21). *E. coli topA*-null mutants are not viable at low temperatures (<30°C) (22, 35). Mutants with a *topA* deletion have increased sensitivity to high temperatures (>50°C) and oxidative challenges because of defects in transcription of stress response genes needed for survival (6, 29, 30, 39).

Compounds that shift the topoisomerase cleavage-religation equilibrium toward DNA cleavage, resulting in an increased or stabilized covalent complex in vivo, have been found to be effective antibacterial and anticancer therapeutic agents (23, 25, 28). These topoisomerase-targeting compounds are often referred to as topoisomerase poisons (13, 19). However, compounds that act as topoisomerase poisons specific for type IA DNA topoisomerases have not been identified. Every bacterial genome encodes a topoisomerase I (11), which would be vulnerable to perturbation during the catalytic cycle of DNA cleavage and religation. Bacterial topoisomerase I should be utilized as a target for development of new classes of antibacterial drugs to combat multidrug-resistant bacteria (38). The validity of targeting type IA topoisomerases in antibacterial drug development was proven with the identification and characterization of inducible *Yersinia pestis* and *E. coli* topoisomerase I mutants that could cause rapid bacterial cell death due to the accumulation of topoisomerase I cleavage complex (4, 5, 34). These cell-killing mutations of bacterial topoisomerase I serve as models for the potential bactericidal drugs that target type IA topoisomerases.

Quinolones achieve killing of bacterial cells by first stabilizing the covalent intermediate complex between bacterial type IIA topoisomerases and the cleaved DNA during the catalytic cycle (7). In *E. coli*, DNA gyrase is the primary target of fluoroquinolones, while topoisomerase IV is a contributing factor to susceptibility (14, 17). The events that follow the stabilization of the covalent topoisomerase complex, leading to bacterial cell death, remain to be elucidated. Previous studies on the effect of DNA replication and protein synthesis on quinolone-mediated cell death suggest that there may be multiple pathways involved, depending on the individual quinolone structure and growth conditions (8, 20, 27, 44). More recent studies suggest that at least part of the bactericidal action of quinolones can be attributed to oxidative damage from reactive oxygen species (10, 12, 18). It is not clear if this oxidative-damage cell death pathway is dependent on the quinolone structure. In this study, the mutant bacterial topoisomerase I proteins that mediate cell killing through accumulation of cleavage complex were utilized to test whether the induction of the oxidative damage cell death pathway is a direct consequence of stabilized covalent protein-DNA complex on the chromosome.

Detection of hydroxyl radicals following induction of mutant bacterial topoisomerase I. *E. coli* strain BW117N (Table 1) has a mutant *Y. pestis* topoisomerase I gene encoding YpTOP-D117N under the control of the BAD promoter inserted into the chromosome (5). Induction of YpTOP-D117N with arabinose resulted in a 10^4- to 10^5-fold decrease in viability, while induction of chromosomally integrated wild-type YpTOP in *E. coli* strain BWYTOP had no significant effect on viability (5). Overnight cultures of BW117N and BWYTOP prepared in LB medium with 2% glucose were inoculated (1:100) into fresh LB medium. At the exponential phase ($A_{600} \sim 0.4$), arabinose (0.0002%) was added to the culture to induce the expression of recombinant proteins. Controls for each strain had 2% glucose to suppress the expression of YpTOP or YpTOP-D117N. After 2.5 h of induction, cells were treated with the fluorescent reporter dye 3′-(*p*-hydroxyphenyl) fluorescein (HPF) to detect the formation of hydroxyl radicals (33) in a FACScan flow cytometer (Becton Dickinson). Hydroxyl radical formation could be detected following induction of mutant YpTOP-D117N by arabinose for 2.5 h (Fig. 1A). Induction of wild-type YpTOP from *E. coli* strain BWYTOP did not result in significant hydroxyl radical formation. Hydroxyl radical formation from induction of mutant YpTOP-D117N is dependent on the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or construction
E. coli strains		
BW27784	DE(*ara*BAD)*567* DE(*rha*BAD)*568* DE(*araFGH*) Φ(Δ*araEp*P_{CP18}-*araE*)	Yale *E. coli* Genetic Stock Center (16)
BWYTOP	BW27784 with chromosomally integrated *Y. pestis* topoisomerase I gene (YpTOP), Cm^r	5
BW117N	BW27784 with chromosomally integrated YpTOP-D117N gene, Cm^r	5
JW2514-4	*iscS776*(del)::Kan; Keio collection	Yale *E. coli* Genetic Stock Center (1)
TA001	BW27784 *iscS776*(del)::Kan	P1(JW2514-4) × BW27784 (this study)
Plasmids		
pETOP	Wild-type *E. coli* topoisomerase I gene (EcTOP) under the control of the BAD promoter in high copy no.	4
pETOPSV	Mutant derivate of pETOP encoding EcTOP-G116S/M320V	3
pAYTOP	Wild-type YpTOP under the control of the BAD promoter in medium copy no.	4
pAYTOP128	Mutant derivative of pAYTOP encoding YpTOP with G122S, M326V, and A383P mutations	4

concentration of arabinose (Fig. 1B). Another mutant bacterial topoisomerase I, *E. coli* topoisomerase I (EcTOP-G116S/M320V), which contains G116S and M320V mutations (3) and is encoded by pETOPSV, is also bactericidal when overexpressed but is not as lethal as the YpTOP-D117N mutant. The multicopy pETOPSV plasmid could therefore be maintained in the presence of 2% glucose in the growth medium to suppress expression from the BAD promoter (3), while YpTOP-D117N, due to its higher lethality, cannot be expressed and maintained in high-copy-number plasmids in *E. coli* (5). Induction of the mutant *E. coli* topoisomerase I EcTOP-G116S/M320V in BW27784 with arabinose also resulted in significant production of hydroxyl radicals detectable by HPF (Fig. 2). Similar results (data not shown) were obtained for induction of mutant *Y. pestis* topoisomerase I containing analogous G122S and M326V mutations and encoded by plasmid pAYTOP128, the first isolated mutant bacterial topoisomerase I with the cell-killing phenotype (4). Levels of hydroxyl radicals induced by two different concentrations of norfloxacin were also determined for comparison (Fig. 2C).

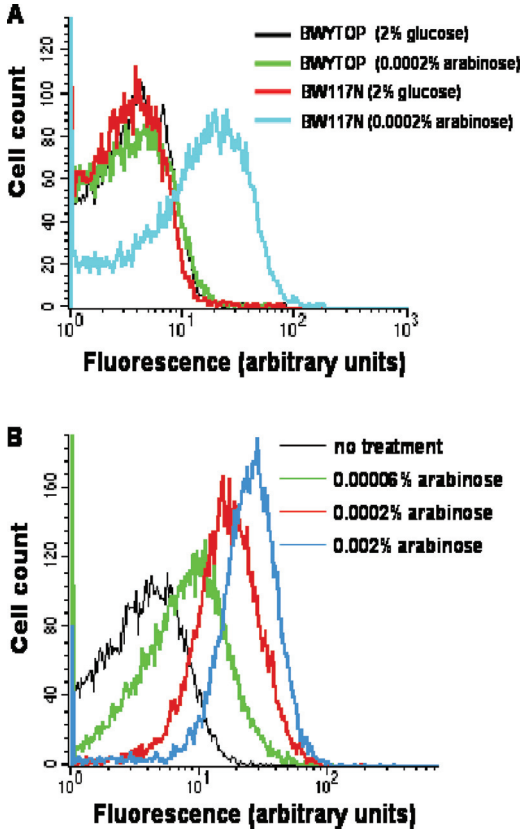

FIG. 1. Hydroxyl radical formation in *E. coli* following accumulation of topoisomerase I cleavage complex. (A) Representative fluorescence population distributions of cultures of BWYTOP and BW117N in the presence of 2% glucose or after addition of 0.0002% arabinose. (B) Effect of different arabinose concentrations on hydroxyl radical formation from induction of YpTOP-D117N in BW117N.

Suppression of cell killing by an iron chelator and an *iscS* mutation. Hydroxyl radicals are generated in the Fenton reaction between free ferrous ions and hydrogen peroxide (37). To demonstrate that hydroxyl radical formation contributes to the cell killing events initiated by accumulation of bacterial topoisomerase I covalent complex, the iron chelator 2,2′-dipyridyl was added to the growth medium along with arabinose when YpTOP-D117N was induced in BW117N. This resulted in suppression of hydroxyl radical formation after induction of mutant YpTOP-D117N in *E. coli* BW117N (Fig. 3A). The reduction of hydroxyl radical formation due to the presence of the iron chelator correlated with an up-to-100-fold increase in cell survival rate (Fig. 3B). Western blot analysis (see Fig. S1 in the supplemental material) showed that the effect of the iron chelator was not due to change in the level of mutant topoisomerase I protein.

The *iscS* gene in *E. coli* codes for cysteine desulfurase and is required for the biosynthesis of Fe-S clusters (32). Previous studies showed that *E. coli* strains with Δ*iscS* have increased resistance to norfloxacin, supporting the role of ferrous iron from Fe-S clusters in generating hydroxyl radicals for the cell death pathway (10). The Δ*iscS* mutation was introduced into strain BW27784 by transduction, resulting in strain TA001. Induction with arabinose showed that viability following expression of mutant YpTOP from pAYTOP128 (viability [ratio of counts of viable cells treated with 0.0002% arabinose to counts of untreated cells], 0.50 ± 0.05 [mean ± standard er-

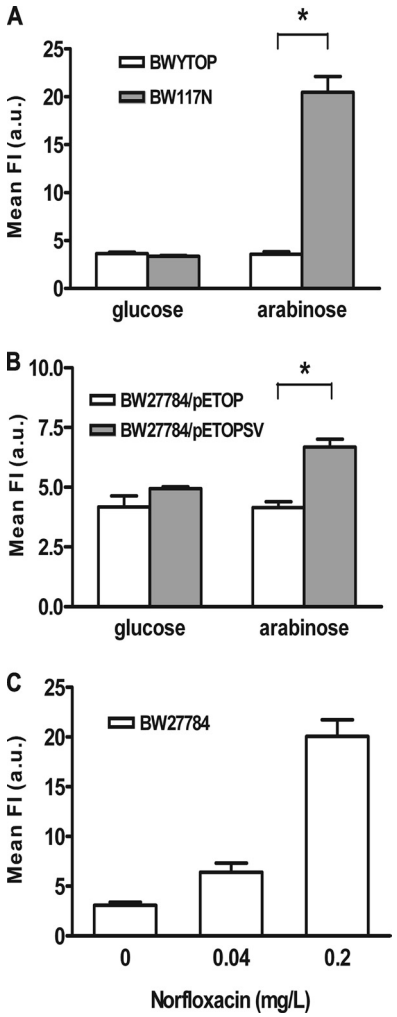

FIG. 2. Quantitation of hydroxyl radicals in BW117N (A) and BW27784/pETOPSV (B) cells, expressing *Y. pestis* and *E. coli* mutant topoisomerase I, and in BW27784 cells treated with norfloxacin (C). Hydroxyl radicals from 20,000 cells were measured by HPF following accumulation of cleavage complex by 0.0008% arabinose or in the presence of 2% glucose as a control. Fluorescence measurements of the cells containing wild-type topoisomerase I (BWYTOP and BW27784/pETOP) are also shown for comparison. Mean fluorescence intensity, expressed in arbitrary units (a.u.), is the level of hydroxyl radical at 2.5 h (BW117N and BW27784) or 3 h (BW27784/pETOPSV) after addition of arabinose or norfloxacin. Data are means plus standard errors of the means from at least triplicate experiments. *, $P < 0.05$.

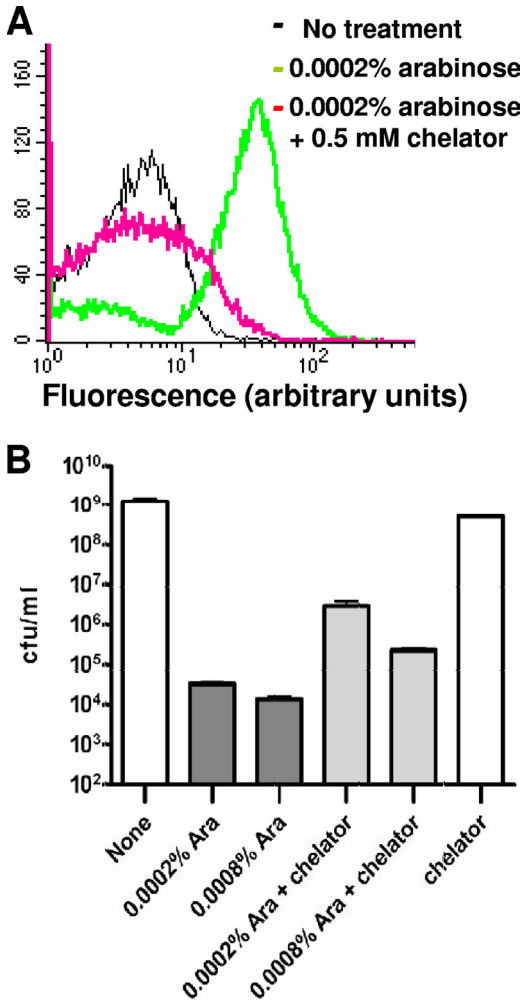

FIG. 3. Effect of the iron chelator 2,2′-dipyridyl on hydroxyl radical formation (A) and relative cell viability (B) following induction of mutant *Y. pestis* topoisomerase I. *E. coli* BW117N was treated with arabinose in the absence and presence of 0.5 mM 2,2′-dipyridyl. CFU per ml were determined at 3 h after addition of arabinose. The results (B) are means and standard errors from triplicate experiments.

ror]) and of mutant EcTOP from pETOPSV (0.24 ± 0.06) was greatly enhanced in TA001 compared with BW27784 (viability, $[2.2 \pm 1.3] \times 10^{-4}$ and $[2.9 \pm 0.3] \times 10^{-5}$, respectively). This further supported the role of hydroxyl radicals generated from ferrous ions of Fe-S clusters in the cell killing mechanism of topoisomerase I cleavage complex accumulation.

Synergistic cell killing by topoisomerase I and gyrase cleavage complex. In order to test whether targeting bacterial topoisomerase I and gyrase simultaneously would enhance the bactericidal action, low levels of arabinose (to induce expression of recombinant mutant YpTOP) and the fluoroquinolone norfloxacin (to induce cleavage complex accumulation by DNA gyrase) were used to treat *E. coli* BW117N expressing the mutant YpTOP-D117N. The fractional inhibitory concentration index cannot be calculated with the arabinose treatment for evaluation of synergy. Nevertheless, synergy from two compounds can be determined if there is a $\geq$2-$\log_{10}$-CFU/ml decrease in viable counts compared to counts with the more active compound alone in a time-kill analysis (2, 15). In contrast, the effect of two treatments will be additive only if there is a <10-fold increase in killing (15). The results of time-kill studies (Table 2) showed that the combination treatment of relatively low concentrations of arabinose to induce the topoisomerase I cleavage complex and norfloxacin to induce the gyrase cleavage complex resulted in a >2-$\log_{10}$-CFU/ml decrease in viability of *E. coli* BW117N compared with viability after each treatment alone.

Oxidative damage is a consequence of covalent protein-DNA complex accumulation. From genetic studies, it has been shown that quinolone treatment of *E. coli* induces the SOS

TABLE 2. Synergistic effects of gyrase inhibitor and topoisomerase I cleavage complex on *E. coli* viability determined in time-kill study

Treatment	Log CFU/ml[a]	
	1 h	2 h
None	8.30 ± 0.04	8.95 ± 0.04
0.00005% arabinose	6.56 ± 0.16	5.94 ± 0.08
0.2 mg/liter norfloxacin	6.78 ± 0.16	5.96 ± 0.11
0.00005% arabinose + 0.2 mg/liter norfloxacin	4.14 ± 0.08	3.46 ± 0.09

[a] Viable counts of BW117N at 1 or 2 h after the addition of 0.2 mg/liter norfloxacin and/or induction of topoisomerase I cleavage complex were determined by serial dilutions and plating on LB plates with 2% glucose and chloramphenicol. The averages and standard errors of results from three experiments are shown.

response via the double-strand break recombination repair pathway with RecBC-dependent loading of RecA (24, 26). Our previous work showed that bacterial topoisomerase I cleavage complexes accumulated on chromosomal DNA are also converted to double-strand breaks to be processed by RecBC (36). This study demonstrates that besides the similarity in processing of the covalent protein-DNA complex, type IA and type IIA topoisomerase cleavage complexes also share a common bactericidal pathway. Based on studies of the effects of DNA replication and protein synthesis inhibition, it was proposed that multiple pathways of cell killing are involved in quinolone action, and the individual quinolone structure may be a factor for certain pathways (8, 20, 27, 44). Hydroxyl radical formation has recently been implicated in the cell death caused by bactericidal antibiotics, including quinolones (10). The results obtained here with the mutant *Y. pestis* and *E. coli* topoisomerase I models suggest that the oxidative damage pathway of cell killing induced by quinolones is likely to be a direct consequence of covalent protein-DNA complex accumulation and is not dependent on the individual quinolone structure. The events that occur after covalent protein-DNA complex accumulation that result in hydroxyl radical formation from the Fenton reaction remain to be fully elucidated. The *Y. pestis* and *E. coli* topoisomerase I mutants employed in this study could provide a useful model for further studies of this bactericidal pathway.

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