DNA topoisomerases are important targets in anticancer and antibacterial therapy because drugs can initiate cell death by stabilizing the transient covalent topoisomerase-DNA complex. In this study, we employed a method that uses CsCl density gradient centrifugation to separate unbound from DNA-bound GyrA/ParC in Escherichia coli cell lysates after quinolone treatment, allowing antibody detection and quantitation of the covalent complexes on slot blots. Using these procedures modified from the in vivo complexes of enzyme (ICE) bioassay, we found a correlation between gyrase-DNA complex formation and DNA replication inhibition at bacteriostatic (1 × MIC) norfloxacin concentrations. Quantitation of the number of gyrase-DNA complexes per E. coli cell permitted an association between cell death and chromosomal gyrA-DNA complex accumulation at norfloxacin concentrations greater than 1 × MIC. When comparing levels of gyrase-DNA complexes to topoisomerase IV-DNA complexes in the absence of drug, we observed that the gyrase-DNA complex level was higher (~130-fold) than that of the topoisomerase IV-DNA complex. In addition, levels of gyrase and topoisomerase IV complexes reached a significant increase after 30 min of treatment at 1 × and 1.7 × MIC, respectively. These results are in agreement with gyrase being the primary target for quinolones in E. coli. We further validated the utility of this method for the study of topoisomerase-drug interactions in bacteria by showing the gyrase covalent complex reversibility after removal of the drug from the medium, and the resistant effect of the Ser83Leu gyrA mutation on accumulation of gyrase covalent complexes on chromosomal DNA.

Methods that use antibodies against topoisomerases are specific for detection of topoisomerase-DNA complexes. These include the band depletion assay (14), the trapped in agarose DNA immunostaining (TARDIS) assay (4), and the in vivo complexes of enzyme (ICE) bioassay (33). The band depletion assay specifically quantitates topoisomerase-DNA complexes based on the slower electrophoretic mobility of the complexes compared to that of free topoisomerase, but the actual complexes are not visualized. In contrast, the TARDIS assay directly visualizes and quantitates topoisomerase-DNA complexes in single cells by immunofluorescence using adduct-specific antibodies. In the TARDIS assay, cells are embedded in agarose and subjected to salt-detergent extraction prior to immunodetection. The ICE bioassay and the TARDIS assay are more sensitive than the band depletion assay. Here we describe a method that is modified from the ICE bioassay. The method achieves trapping of endogenous topoisomerase-DNA complexes by cell lysis in the presence of Sarkosyl, followed by separation of the complexes from free topoisomerase protein using a CsCl density gradient fractionation method that relies on the different densities of DNA-bound topoisomerase and free topoisomerase. Adapting this method for the first time for study of topoisomerase complexes in bacteria, we were able to quantitate.

Received 5 June 2012 Returned for modification 5 July 2012 Accepted 27 July 2012 Published ahead of print 6 August 2012 Address correspondence to Yuk-Ching Tse-Dinh, yukching.tsedinh@fiu.edu. * Present address: Yuk-Ching Tse-Dinh, Department of Chemistry and Biochemistry, Florida International University, Miami, Florida, USA.

Supplemental material for this article may be found at http://aac.asm.org.
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AAC.01182-12
endogenous gyrase- and topoisomerase IV-DNA covalent complexes formed in *E. coli* following norfloxacin treatment and correlate the results to MIC, DNA synthesis inhibition, and cell death.

**MATERIALS AND METHODS**

**Treatment with quinolones.** A culture of *E. coli* strain BW27784 \(\Delta araD-arab\)567 \(\Delta inZ4787(\Delta rnmB-3) \lambda \Delta (araH-araF)570(\Delta FRT)\) \(\Delta araE\rho-532\Delta araE\rho\) strain \(\Delta (ralD-ralB)568\) was grown to an optical density of 0.4 (OD\text{600} = 0.4) and divided into 50-ml aliquots for each drug treatment. Norfloxacin or nalidixic acid was added to the divided cultures at concentrations indicated in the figure legends. Norfloxacin- or nalidixic acid-containing cultures were then further incubated at 37°C with shaking (225 rpm) for various times. Cells were collected by centrifugation (4,000 rpm for 10 min) at 20°C.

In time course experiments, 50 ml of culture was collected immediately after the addition of norfloxacin (time zero). Norfloxacin was added to the remaining culture, and aliquots of 50 ml of culture were collected for lysis. In assay for reversal of the covalent complex, 20-50 ml aliquots of culture were collected by centrifugation after 30 min of norfloxacin treatment (0.2 \(\mu\)g/ml); one aliquot was lysed, and the other aliquot was suspended in norfloxacin-free medium before further incubation for another 30 min.

**Preparation of bacterial cell lysates.** Following collection of the cells by centrifugation, pellets (from a 50-ml culture) were resuspended in 2 ml of lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 0.3 M NaCl, 1% Sarkosyl), followed by immediate addition of 30 KU of rLysozyme (Novagen) and approximately 0.5 g of glass beads (Sigma-Aldrich). Lysis was carried out at 37°C for 45 min with shaking (225 rpm) and ended by incubation at room temperature for 15 min with rapid shaking (3,000 rpm). We found that lysis at 37°C was crucial for good yield of covalent complex and that lysis at 4°C or 0°C yielded negative results. Lysates were cleared by centrifugation at 30,000 rpm for 30 min at 20°C in a Beckman Optima LE-80K ultracentrifuge with a Beckman Type 50 Ti rotor.

**Preparation of CoCl density gradients.** Cesium chloride (CoCl) density gradients were used to separate free proteins from DNA based on their different densities. A stock solution of cesium chloride was made by dissolving 63.2 g of CoCl (Affymetric-USB Corporation) in 36.8 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). This stock solution with a density of 1.86 g/ml was used to prepare four CoCl solutions with densities of 1.82 g/ml, 1.72 g/ml, 1.50 g/ml, and 1.37 g/ml. Two milliliters of every solution was carefully layered in a polyallomer tube (14 by 89 mm) starting from the solution with the highest density to the solution with the lowest density. Bacterial cell lysates (approximately 2 ml) were loaded on top of the gradients, and tubes were topped off with mineral oil. Centrifugation was carried out at 31,000 rpm for 16 h at 20°C with a Beckman SW41Ti rotor. A 22-gauge needle was then used to pierce the tubes containing the gradient in the bottom. Fractions of 1 ml were collected from the bottom of the tubes as indicated in the figure legends.

**DNA concentration measurements.** The concentration of double-stranded DNA (dsDNA) was determined in every fraction by a fluorocence assay using Quant-iT PicoGreen dsDNA reagent from Invitrogen as described by the manufacturer. Briefly, fractions were diluted (1:1,000) in TE buffer before being mixed with equal volumes of PicoGreen working solution. Samples of salmon sperm dsDNA (Sigma-Aldrich) for a standard curve were prepared in parallel in TE buffer. The standard curve and the diluted CoCl density gradient fractions were incubated with PicoGreen reagent for 5 min at room temperature in a 96-well black plate. The final volume of the assay was 200 \(\mu\)l. Fluorescence was measured using the BioTek Synergy HT plate reader with excitation at 485 nm and emission at 528 nm.

**Topoisomerase immunodetection by slot blotting.** In order to compare the levels of topoisomerase linking to chromosomal DNA from cultures obtained under different experimental conditions, equal amounts of DNA present in the CoCl gradient fractions to be compared were loaded onto the membrane. The volumes required to load equal DNA amounts were calculated according to the DNA concentrations as described previously (11). Samples were diluted with an equal volume of 25 mM sodium phosphate buffer (pH 6.5) and loaded on a preloaded nitrocellulose membrane using a Bio-Dot SF (Bio-Rad) apparatus. Following loading, the membrane was blocked for 1 h at room temperature in 1% bovine serum albumin (Fisher Scientific) made in TBST buffer (20 mM Tris-HCl [pH 7.5], 0.14 M NaCl, 0.1% Tween 20) for gyrase blots or in 5% skimmed milk for topoisomerase IV blots. Blocked membranes were incubated overnight at 4°C with a commercial rabbit polyclonal antibody against gyrase subunit GyrA (1:250, Abcam) or with a rabbit polyclonal antibody against subunit ParC (1:10,000) used to detect topoisomerase IV. The anti-ParC antibody was a kind gift from Kenneth Marians at Sloan-Kettering Institute. The membrane was then washed for 15 min with TBST buffer prior to 1 h of incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (diluted 1:3,000 for GyrA and ParC blots) (Santa Cruz Biotechnology). Following incubation with the secondary antibody, the membrane was washed for 15 min with TBST buffer, followed by three additional washes of 5 min each. Finally, the membrane was incubated with ECL Plus reagent from PerkinElmer as instructed by the manufacturer. An Amersham Hyperfilm ECL X-ray film was exposed (2 to 3 min) to the membrane and developed.

**Quantitation of covalent complex.** CoCl density gradient fractions were analyzed by slot blotting along with 2-fold serial dilutions of known amounts of purified gyrase (New England BioLabs) or topoisomerase IV (Topogen) protein as standards. Blots were imaged using the AlphaInnotech Chemilumager 5500 system, and protein band densities were analyzed using AlphaEaseFC software. The amount of either gyrase or topoisomerase IV protein in the loaded CoCl density gradient fractions was determined using the standard curve. The amount of covalent complex found in the chromosomal DNA fractions was expressed as ng of gyrase or topoisomerase IV divided by \(\mu\)g of DNA. For quantification of the gyrase or topoisomerase IV signal in gradient fractions from untreated samples, DNA amounts 2 to 3 times larger were loaded simultaneously with the rest of the fractions so that a signal sufficient for quantification could be obtained.

**Construction of quinolone-resistant *E. coli* strain with GyrA mutation S83L.** The gyra37 mutation at amino acid 83 (S83L) conferring nalidixic acid resistance was introduced into *E. coli* strain BW27784 by P1 transduction using *E. coli* strain UB1005 (from the Coli Genetic Stock Center at Yale University) as the donor. Selection of the mutants was done on agar plates containing 45 \(\mu\)g/ml of nalidixic acid. The gyra37 mutation was confirmed by sequencing of the quinolone resistance determinant region amplified by PCR with primers 5’-ATG AGC GAC CTT GGG AGA GAA ATT ACC GG-3’ and 5’-CCA TCA GCC CTT CAA TGC CTA GTG CTT C-3’. DNA synthesis measurements. *E. coli* cultures were grown up to exponential phase (OD\text{600} = 0.4) in M9 medium supplemented with 2% glucose and 1% Casamino Acids. Norfloxacin was then added, and aliquots of 200 \(\mu\)l were taken and transferred to tubes containing 1 \(\mu\)Ci of \([\text{3H}]\)thymidine. These samples were further incubated for 2 min at 37°C. Incorporation of radioactivity was terminated with the addition of 1 ml of cold 10% trichloroacetic acid. Acid precipitates were collected in Whatman GF/A filters and washed with cold 1 N HCl and 95% ethanol. Filters were dried, and radioactivity was measured by liquid scintillation counting. The rate of DNA synthesis was measured as the radioactivity remaining on the filters and expressed as a percentage of the untreated control at time zero.

**RESULTS**

A method for isolation and quantitation of endogenous topoisomerase-DNA covalent complex was developed in *E. coli* based on the ICE assays for mammalian cells (30, 35) and yeast (11, 12, 15). The method was used to study a correlation between covalent
complex formation and MIC, DNA synthesis inhibition, and cell death. The method described here consists of two steps. In the first step, topoisomerase-DNA complex formation was isolated from *E. coli* cell lysates using a cell fractionation method, and in the second step, the complexes were detected and quantitated by slot blotting. Prior to isolation and quantitation of the complexes, norfloxacin was added to an *E. coli* culture to stabilize endogenous topoisomerase-DNA complex during exponential growth phase, while topoisomerase was actively engaged in the DNA cleavage-religation steps of the catalytic cycle. Following stabilization of the covalent complex with norfloxacin, rapid cell lysis was carried out in the presence of Sarkosyl, which served to trap the topoisomerase protein covalently bound to DNA by protein denaturation and dissociate the noncovalently bound topoisomerase protein (free topoisomerase). In the isolation step, the denatured cell lysates were subjected to a cell fractionation method in which separation of the free topoisomerase from the DNA-bound complex was achieved by sedimentation in a cesium chloride density gradient that resolves protein from DNA based on their individual densities. DNA has a higher density than proteins; thus, it sediments closer to the bottom of the gradient, whereas free topoisomerase protein sediments closer to the top of the gradient. DNA-bound topoisomerase sediments to the position of DNA near the bottom of the gradient. Fractionation of the gradients (from bottom to top) was followed by detection and quantitation of topoisomerase in the bottom DNA fractions as covalent protein-DNA complex with antibody against topoisomerase using slot blotting. For quantitation of the topoisomerase signal, known amounts of topoisomerase were loaded on the blots as standards. This method would capture topoisomerase linked to DNA from both double-stranded and single-stranded DNA breaks, as shown previously for eukaryotic type I and type II topoisomerases (11, 32).

Figure 1 shows representative slot blots of dose response experiments with *E. coli* strain BW27784 treated for 30 min in the absence or presence of different norfloxacin concentrations (0.03, 0.06, 0.10, 0.15, or 0.20 μg/ml) of norfloxacin. Cells were lysed in the presence of detergent and topoisomerase-DNA complexes separated from free topoisomerase by sedimentation in a CsCl density gradient. Gradients were fractionated from the bottom (high density) to the top (low density) and loaded on a slot blot. (A) Representative slot blot probed with an anticyrase antibody. (Left) CsCl density gradient fractions 1 to 4 collected from the bottom portion of the gradient; (right) CsCl density gradient fractions 6 to 10 collected from the top portion of the gradient. The graph shows quantitation of results from four independent experiments. The data shown represent the averages and standard errors (***, P < 0.001 compared to control). (B) Representative slot blot probed with an anti-ParC antibody. (Left) CsCl density gradient fractions 1 to 4 collected from the bottom portion of the gradient; (right) CsCl density gradient fractions 6 to 10 collected from the top portion of the gradient. The graph shows quantitation of results from three independent experiments. The data shown represent the averages and standard errors (*, P < 0.05 compared to control).

FIG 1 Isolation and quantitation of endogenous topoisomerase-DNA complexes formed in *E. coli* following norfloxacin treatment. An exponentially growing culture of *E. coli* strain BW27784 was incubated for 30 min with 0, 0.03, 0.06, 0.10, 0.15, or 0.20 μg/ml of norfloxacin. Cells were lysed in the presence of detergent and topoisomerase-DNA complexes separated from free topoisomerase by sedimentation in a CsCl density gradient. Gradients were fractionated from the bottom (high density) to the top (low density) and loaded on a slot blot. (A) Representative slot blot probed with an anticyrase antibody. (Left) CsCl density gradient fractions 1 to 4 collected from the bottom portion of the gradient; (right) CsCl density gradient fractions 6 to 10 collected from the top portion of the gradient. The graph shows quantitation of results from four independent experiments. The data shown represent the averages and standard errors (***, P < 0.001 compared to control). (B) Representative slot blot probed with an anti-ParC antibody. (Left) CsCl density gradient fractions 1 to 4 collected from the bottom portion of the gradient; (right) CsCl density gradient fractions 6 to 10 collected from the top portion of the gradient. The graph shows quantitation of results from three independent experiments. The data shown represent the averages and standard errors (*, P < 0.05 compared to control).
higher norfloxacin concentration (0.1 μg/ml of norfloxacin, equivalent to 1.7× MIC) than did gyrase-DNA complexes to reach a statistically significant increase. The levels of topoisomerase IV-DNA complexes were at least 150-fold lower than those of gyrase-DNA complexes in the untreated control and every norfloxacin-treated sample.

Inhibition of DNA synthesis by quinolones has been shown to take place within 1 to 5 min after addition of the gyrase inhibitor to E. coli cells (23, 29). The very early time course of gyrase-DNA complex formation was examined along with DNA synthesis inhibition at 0.06 μg/ml of norfloxacin (1× MIC). An increase in the level of gyrase-DNA complex was observed along with a 25% reduction of rate of DNA synthesis as early as 1 and 5 min after treatment (Fig. 2). In contrast to rapid inhibition of DNA synthesis, reduction in cell viability was not observed up to 2 h after treatment with 0.06 μg/ml of norfloxacin (Fig. 3).

The number of drug-stabilized gyrase-DNA complexes that are found in the chromosome of an E. coli cell at a given drug concentration has not been determined previously. This could be estimated in our study using known amounts of gyrase protein as standards on the slot blot. In our experiments, gyrase-DNA complexes are expressed as ng of gyrase per μg of DNA. In order to convert our data into molecules of gyrase-DNA complex per cell, we applied the following formula: (G of gyrase)/374,000 Da of gyrase)/(1 × 10^-6 g of DNA/3 × 10^9 Da of DNA). G of gyrase corresponds to the amount of gyrase protein (molecular mass = 374,000 Da) found to be associated with each μg of DNA (molecular mass of E. coli chromosome = 3 × 10^9 Da) from the bottom of the gradient. The results from quantitation of four independent experiments are shown in Table 1. The number of gyrase complexes increased 3.8-fold at 30 min following treatment with 0.06 μg/ml (1× MIC) of norfloxacin. This drug concentration was sufficient for growth inhibition but not for cell death (Fig. 3). Further increase of the norfloxacin concentration to 0.1 μg/ml resulted in an ~6.2-fold increase in the number of gyrase-covalent complexes to around 36 per cell (Table 1), corresponding to close to 99% loss of viability at 2 h (Fig. 3). The molecular mass of 374,000 Da corresponds to the gyrase complex A2B2. Therefore, the formula assumes double-strand DNA breaks, although quinolones are known to trap both single- and double-strand breaks by gyrase (29). The number of chromosomal breakage sites would be higher if some of the quantitated GyrA-DNA complexes were found at single-strand breaks.

Quinolones are known to reversibly stabilize covalent gyrase-DNA complexes. Reversibility of the covalent gyrase-DNA complex upon drug removal was assessed. Following treatment with 0.20 μg/ml of norfloxacin for 30 min, half of the treated and control cultures were lysed immediately, while the remaining halves of the cultures were centrifuged, resuspended in fresh medium, and incubated for another 30 min at 37°C before lysis. The slot blot containing the bottom DNA fractions from a representative

### Table 1

<table>
<thead>
<tr>
<th>Norfloxacin concn (μg/ml)</th>
<th>No. of gyrase-DNA covalent complexes per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.84 ± 0.10</td>
</tr>
<tr>
<td>0.03</td>
<td>10.05 ± 0.32</td>
</tr>
<tr>
<td>0.06</td>
<td>21.94 ± 0.06</td>
</tr>
<tr>
<td>0.10</td>
<td>35.99 ± 5.30</td>
</tr>
<tr>
<td>0.15</td>
<td>49.71 ± 0.43</td>
</tr>
<tr>
<td>0.20</td>
<td>72.11 ± 0.92</td>
</tr>
</tbody>
</table>

*Exponentially growing cultures of E. coli strain BW27784 were incubated for 30 min in the absence or presence of norfloxacin. Following treatment, cells were lysed and covalent complexes isolated from the cell lysates as described in the legend to Fig. 1. The number of gyrase molecules was determined by loading known amounts of gyrase protein as standards on the slot blots. The results represent the means and SEM of four independent experiments.*
experiment is shown in Fig. 4A. For quantification, the signal of the covalent gyrase-DNA complex from each cell lysate was normalized with the signal of the free gyrase protein in the top fractions. Results from three independent experiments showed that the normalized signal from the covalent gyrase-DNA complex was diminished 2.8-fold (mean ± standard error of the mean [SEM]) following removal of the drug (Fig. 4B). This result indicated that the signal of the covalent complex detected by this method was at least partially reversible.

A number of mutations in the quinolone resistance determinant region of gyrase protein have been characterized and shown to increase the MICs as a result of alteration in the ability of quinolones to bind to gyrase (31, 38, 39). The most common mutations in this region of \textit{E. coli} GyrA protein are located at amino acids 83 and 87. In order to assess the applicability of the method described here in the molecular characterization of quinolone-resistant bacteria, we introduced the \textit{gyrA}37 mutation at amino acid 83 (Ser to Leu), known to confer resistance to quinolones. Covalent complex formation was examined in the resulting quinolone-resistant \textit{E. coli} strain after 30 min of incubation in the absence and presence of 0.20 μg/ml of norfloxacin (0.001). A.U., arbitrary units.

To demonstrate that this method is applicable to other gyrase poison inhibitors, \textit{E. coli} strain BW27784 was treated with the less potent gyrase inhibitor nalidixic acid and analyzed. The immunoblot showed that the gyrase-DNA covalent complex could also be detected readily following nalidixic acid treatment (Fig. 6).
DISCUSSION

Methods for specific detection and quantification of endogenous topoisomerase-DNA covalent complexes in mammalian cells (4, 14, 32, 33) and yeast (12) have been previously described. However, to our knowledge, no such method has been reported for bacteria. Here we describe a method for isolation and quantitation of endogenous topoisomerase-DNA covalent complexes accumulated on E. coli chromosomal DNA following treatment with quinolones. In addition, we explored the potential of this method to reveal aspects of cleavage complex formation in the mechanism of quinolone-mediated cell death.

In this method, which is modified from the ICE bioassay (33), endogenous topoisomerase-DNA covalent complexes stabilized in E. coli with quinolones were trapped by cell lysis in the presence of an ionic detergent. Isolation of the trapped covalent complexes from cell lysates was then carried out using a cell fractionation method. Following fractionation in a CsCl density gradient, complexes were detected and quantitated by slot blotting using antibodies against topoisomerase.

Using this method, we first investigated a correlation between topoisomerase-DNA complex formation and MIC. We found a nearly linear increase of both gyrase- and topoisomerase IV-DNA complexes in response to increasing concentrations of norfloxacin (from 0.5 MIC to up to 3 MIC) (Fig. 1). Previously, associations between complex formation and MIC have been done by indirect measurements of inhibition of DNA synthesis (29) and chromosomal fragmentation (23). Our results correlate MIC to covalent complex formation by a direct measure of the complexes. Further, these results are consistent with the other indirect methods, as discussed below, for the correlation of covalent complex formation to DNA synthesis inhibition.

A rapid increase (2-fold), within 1 min, in the levels of gyrase-DNA complexes was accompanied by a moderate decrease (25%) in DNA synthesis at the bacteriostatic norfloxacin concentration (1 MIC) (Fig. 2). The moderate inhibition of DNA synthesis may be due to a sparse distribution of the complexes on the chromosome. Importantly, this rapid increase in complex formation and DNA synthesis inhibition blocked growth after 30 min and 2 h but did not cause cell killing.

Cell killing was observed only after 2 h of treatment with norfloxacin concentrations of >1 MIC (Fig. 3). These results support the general concept that DNA synthesis inhibition correlates with the initial formation of reversible complexes upon addition of quinolones. However, cell killing is the result of a yet-to-be-defined second step that occurs after initial growth blockage (8).

Cell killing has been attributed to chromosome fragmentation (23) and the formation of reactive oxygen species (10, 37). It has been proposed that chromosome fragmentation depends on a suicide factor that requires protein synthesis (8) possibly for the expression of processing activities that remove the gyrase protein from the chromosome, converting the covalent complex into irreversible DNA breaks (23). Cell death would occur if the DNA repair system is inadequate for repair of the exposed DNA breaks. Proteolytic processing of eukaryotic topoisomerases I (5, 42) and II (41) has been shown to occur after treatment with camptothecin and etoposide, respectively. Further studies are needed to investigate the proteolytic or nucleolytic processing of topoisomerase-DNA complexes in bacteria. A number of studies have applied the ICE bioassay to study the repair pathways of drug-stabilized topoisomerase covalent complex in eukaryotic cells (1, 13, 43). The method described here for bacteria could also be applied to identify processing or repair mechanisms of stabilized gyrase-DNA complexes. These bacterial processing or repair mechanisms may be potential targets of new antibiotics for use in combination with gyrase inhibitors.

Using known amounts of gyrase protein as standards in the blots, we were able to determine the number of complexes sufficient for cell killing after treatment with norfloxacin (Table 1). We estimated that there were around 22 gyrase-DNA covalent complexes in each E. coli cell treated with norfloxacin at 1 MIC (0.06 μg/ml). A higher number of gyrase covalent complexes (72 per cell after treatment with 0.2 μg/ml of norfloxacin) was required to observe a moderate reduction in cell viability (Fig. 3). Significant reduction of cell viability required longer incubation time, likely because other physiological events, including the generation of reactive oxygen species (10, 37), may take place prior to cell death.

The levels of gyrase complexes were >150-fold higher than those of topoisomerase IV complexes in lysates of untreated control cells. Moreover, a higher concentration of norfloxacin (1.7 MIC for topoisomerase IV compared to 1 MIC for gyrase) was required to observe a significant increase in the level of topoisomerase IV-DNA complexes. These results are in agreement with gyrase being the primary target for the topoisomerase poison action of norfloxacin in E. coli. This most likely is explained by their different cellular functions. Gyrase plays a more active role than topoisomerase IV during DNA replication to alleviate topological stress ahead of the replication fork (7). In contrast, topoisomerase IV is mainly needed for chromosome segregation at the end of a round of replication (17, 19). In support of this rationale, previous work has shown that in order to inhibit supercoiling and decatenation to the same extent, a higher norfloxacin concentration is needed for inhibition of the latter (19). Gyrase covalent complexes may also be associated with regions of high transcription activity due to the involvement of gyrase in relief of transcription-driven supercoiling (9, 36). The activity of topoisomerase IV is secondary for the relief of superhelical tension (18, 40). Other factors that could account for why gyrase is the primary target of quinolones in E. coli include a higher level of gyrase protein and greater intrinsic sensitivity of gyrase activity to inhibition by quinolones (19). Both of these factors would favor cleavage complex accumulation for gyrase versus topoisomerase IV.

Regarding the cell fractionation method used here, the labor involved in preparing the gradients is minimal; however, some limitations are worth noting: considerable centrifugation time is needed and rotor capacity is limited. This assay is therefore not useful as a primary high-throughput screening assay but could provide important confirmation for compounds postulated to be topoisomerase poisons from results of enzyme assays. This analysis method can clearly differentiate increased accumulation of topoisomerase covalent complexes from other mechanisms of DNA fragmentation. Detection of the covalent topoisomerase-DNA covalent complexes was used to confirm the mode of action of newly identified inhibitors of human topoisomerases I and II (2, 21, 25). Novel lead compounds found to inhibit bacterial topoisomerases in enzyme-based assays could be tested with the method described here to determine if they can lead to accumulation of the topoisomerase-DNA covalent complex on chromosomal DNA of treated bacterial cultures.
ACKNOWLEDGMENT

This research was supported by award number R01AI069313 (to Y.-C.T.-D.) from the National Institute of Allergy and Infectious Diseases.

REFERENCES