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# Comparison of Inhibition of *Escherichia coli* Topoisomerase IV by Quinolones with DNA Gyrase Inhibition

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**In order to examine the inhibitory activities of quinolones against topoisomerase IV, both subunits of this enzyme, ParC and ParE, were purified from *Escherichia coli*. The specific activity of topoisomerase IV decatenation was found to be more than five times greater than that of topoisomerase IV relaxation. Thus, the decatenation activity of topoisomerase IV seems the most relevant activity for use in studies of drug inhibition of this enzyme. Although topoisomerase IV was less sensitive to quinolones than DNA gyrase, the 50% inhibitory concentrations for decatenation were significantly lower than those for type I topoisomerases. Moreover, there was a positive correlation between the inhibitory activity against topoisomerase IV decatenation and that for DNA gyrase supercoiling. These results imply that topoisomerase IV could be a target for the quinolones in intact bacteria and that quinolones could inhibit not only supercoiling of DNA gyrase but also decatenation of topoisomerase IV when high concentrations of drug exist in bacterial cells.**

Four topoisomerases have been isolated from *Escherichia coli* so far: topoisomerase I (39), topoisomerase II (DNA gyrase) (9), topoisomerase III (34), and topoisomerase IV (16). Among these enzymes, topoisomerases I, II, and IV are implicated in the control of the intracellular supercoiling density of chromosomal or plasmid DNA, affecting the efficacy of DNA replication and transcription (4, 6–8, 20, 24, 29, 36, 37). In addition, some topoisomerases are required for the segregation of daughter chromosomes or plasmid DNA (1, 21, 35, 40). Although all of these topoisomerases are capable of changing the topology of DNA by a cleaving and rejoining step, each enzyme appears to possess a favored reaction in vitro. Type I topoisomerases, topoisomerase I and topoisomerase III, show efficient relaxing and decatenating activity, respectively, in the absence of ATP (2, 3, 5, 11, 29, 30, 34). Of the type II topoisomerases, DNA gyrase is unique in its ability to supercoil relaxed DNA in the presence of ATP (9) and topoisomerase IV decatenates catenated DNA in vitro (28). Furthermore, in vitro only DNA gyrase and topoisomerase IV can segregate intact catenated DNA in the presence of ATP (1, 19, 23, 27, 35); however, recent work has suggested that DNA gyrase may not act as a decatenating enzyme in bacterial cells (1, 11). The type II topoisomerases are thought to be essential for bacterial growth because of inducible lethality in temperature-sensitive mutants (16, 17). The type I topoisomerases are not essential for bacterial growth, because their loss can be compensated for by alterations in type II topoisomerase genes (4, 5).

Recently, the two genes that code for the subunits of topoisomerase IV, *parC* and *parE*, have been cloned (16), and strains which overexpress both subunits have been constructed (18, 27). Interestingly, the deduced amino acid sequences of ParC and ParE are homologous to GyrA and GyrB (the two subunits of DNA gyrase), respectively (16). Similar sequences are located especially around the region of DNA gyrase known as the "quinolone resistance-determining region." The quinolone antibacterial agents, in particular the fluoroquinolones, strongly inhibit DNA gyrase, which results ultimately in bacterial death (32). However, the inhibitory activities of quinolones against type I topoisomerases, topoisomerase I (25) and topoisomerase III (2) in *E. coli*, are weak. The inhibitory activities of fluoroquinolones against topoisomerase IV have yet to be fully investigated. The similarity in amino acid sequence between DNA gyrase and topoisomerase IV, especially around sites in the GyrA protein of DNA gyrase at positions known to produce quinolone-resistant DNA gyrases (41), implies that quinolones may be capable of inhibiting the activity of topoisomerase IV as well as against DNA gyrase.

In this study, in order to define the inhibitory activities of quinolones against topoisomerase IV, both subunits of topoisomerase IV, ParC and ParE, were purified, and optimum conditions for the decatenating activity of topoisomerase IV were determined. Inhibitory studies indicated that although topoisomerase IV was less sensitive to quinolones than DNA gyrase, the 50% inhibitory concentrations ($IC_{50}$s) for decatenation were significantly lower than those for type I topoisomerases. In addition, the specific activity of topoisomerase IV for decatenation was more than five times greater than that for relaxation. Finally, we found that there was a good correlation between the inhibitory activities of quinolones against topoisomerase IV decatenation and DNA gyrase supercoiling.

## MATERIALS AND METHODS

**Chemicals.** DU-6859a (31), DV-7751a (38), DR-3355 (levofloxacin) (10), DR-3354 (10), ciprofloxacin (CPFX), sparfloxacin (SPFX), and nalidixic acid (NA) were synthesized in our laboratories (Fig. 1). All other chemicals were purchased from their respective manufacturers and were at least of analytical grade. Quinolone compounds except DR-3355 and DR-3354 were dissolved in 0.1 N NaOH and diluted with distilled water. Other compounds were dissolved directly in distilled water.

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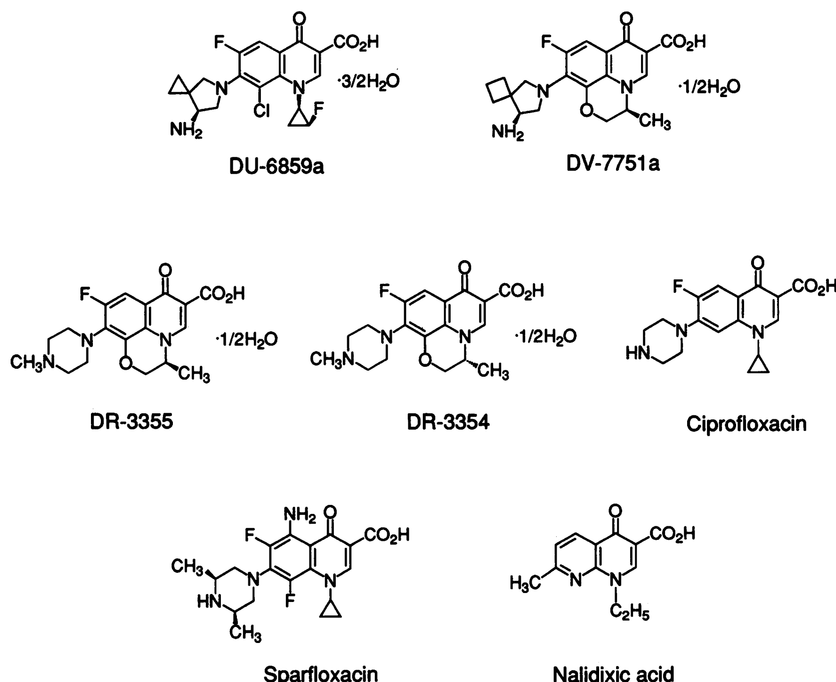


FIG. 1. Chemical structures of quinolone compounds.

**Bacterial strains, plasmids, and culture media.** The *E. coli* strains used in this study were DH1 ($F^-$ *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*) (22) and YN2942 [Δ(*int*-cIII)BAM N::Kan *c*I857Δ(*cro-bioA*)] (14). Supercoiled pBR322 plasmid DNA and kinetoplast DNA (kDNA) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and TopoGEN, Inc. (Columbus, Ohio), respectively. pJK825 and pJK2020 (16) were used for overexpression of ParC and ParE subunits, respectively. Bacteria were grown routinely in Luria-Bertani broth and antibiotic medium 3 (Difco).

**Preparation and manipulation of DNA.** The techniques used for the preparation, manipulation, and transformation of plasmid DNA were carried out as described previously (16).

**Purification of ParC and ParE proteins.** The ParC and ParE proteins of topoisomerase IV were purified from overproducing strains as described previously (18) with minor modifications. All procedures were carried out at 4°C. NaCl was replaced with KCl as the elution solute for each step of purification. Both proteins were detected as distinct protein bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The crude lysate of the ParC- or ParE-overproducing strain was subjected to ammonium sulfate fractionation and then loaded onto a hydroxylapatite column (Seikagaku Kogyo Corp., Tokyo, Japan). The fractions which contained ParC or ParE protein were subjected to Q-Sepharose column chromatography (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden). The last step of molecular sieving by Sephacryl S400 or S200 was omitted. Finally, the ParC or ParE fractions were concentrated with a Q-Sepharose fast flow column and stored at −80°C containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM 2-mercaptoethanol, 300 mM KCl, and 50% glycerol.

**Decatenation of kDNA.** Standard reaction mixtures (20 μl) contained 39 mM Tris-HCl (pH 7.5), 50 μg of bovine serum albumin per ml, 5.8 mM $MgCl_2$, 70 mM KCl (unless indicated otherwise), 1 mM dithiothreitol, 0.5 mM ATP, 0.4 μg of kDNA, appropriate amounts of ParC and ParE (to give 1 U of decatenating activity), and various concentrations of antibacterial agents. The mixtures were incubated at 37°C for 1 h. When the optimum cation concentrations were determined, reaction times were reduced. Reducing reaction times was found to increase the sensitivity of topoisomerase IV to changes in cation concentration. Reactions were terminated by adding 3-μl portions of a solution containing 5% Sarkosyl, 0.0025% bromophenol blue, and 25% glycerol. Reaction results were analyzed by 0.7% agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed under UV light with Polaroid type 665 film. The brightness of bands which corresponded to decatenated monomers of kDNA was traced with a computed densitometer (ACD25-12; ATTO Co. Ltd., Tokyo, Japan). One unit of decatenating activity was defined as the amounts of ParC and ParE proteins required to fully decatenate 0.4 μg of catenated kDNA. The $IC_{50}$s were defined as the drug concentration which reduces the decatenation seen with drug-free controls by 50%.

**Relaxation of supercoiled pBR322 plasmid DNA.** Standard reaction mixtures (20 μl) contained 39 mM Tris-HCl (pH 7.5), 50 μg of bovine serum albumin per ml, 5.8 mM $MgCl_2$, 70 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 0.2 μg of pBR322 DNA, appropriate amounts of ParC and ParE (to give 1 U of relaxing activity), and various concentrations of antibacterial agents. The reactions were processed and analyzed in the same manner as the decatenation assays, except that the $IC_{50}$s were calculated from the decrease in the amount of supercoiled pBR322 DNA. One unit of relaxing activity was defined as the amounts of ParC and ParE proteins required to fully relax 0.2 μg of supercoiled pBR322 DNA.

## RESULTS

**Enzymatic activities of topoisomerase IV.** The ParC and ParE proteins were separately purified to near homogeneity

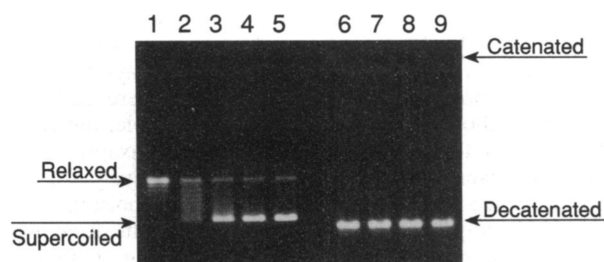


FIG. 2. Relaxation and decatenation activities of topoisomerase IV. Standard reaction mixtures (for relaxing assay) containing 1 U (as a relaxing enzyme) (lane 1) or 0.8 (lanes 2 and 6), 0.6 (lanes 3 and 7), 0.4 (lanes 4 and 8), or 0.2 (lanes 5 and 9) U were incubated with relaxed pBR322 plasmid DNA (lanes 1 to 5) or kDNA (lanes 6 to 9).

(more than 80%) by detecting the ParC and ParE bands by SDS-PAGE. The specific activities of ParC and ParE for decatenation were about $1 \times 10^4$ and $5 \times 10^3$ U/mg of protein, and those for relaxation were $2 \times 10^3$ and $1 \times 10^3$ U/mg of protein, respectively. Thus, the decatenating activity of topoisomerase IV was five times greater than the relaxing activity. This phenomenon is illustrated in Fig. 2, where it can be seen that when ParC and ParE were added to give 1 U of relaxing activity, supercoiled DNA was fully relaxed. If less than 1 U was added, by definition, the supercoiled DNA was no longer fully relaxed. However, full decatenation of kDNA still occurred even when only one-fifth of one "relaxing" unit of topoisomerase IV was added. No topoisomerase activity other than topoisomerase IV was found in the ParC and ParE fractions from the evaluation of relaxing, supercoiling, and decatenating activity with or without ATP (data not shown). Next, the optimum concentration of potassium ion for the decatenating activity of topoisomerase IV was determined. As shown in Fig. 3, the optimum concentration range for potassium cation was around 60 to 90 mM. The concentration of magnesium required was greater than 2 mM (data not shown). From these results, we defined the conditions for the decatenation assay as described in Materials and Methods.

**Inhibitory activities of quinolones and novobiocin against topoisomerase IV.** The effects of quinolones on the decatenating activity of topoisomerase IV are shown in Fig. 4. From the quantitation of bands which corresponded to fully decatenated substrate, $IC_{50}$s for the quinolones were calculated and are shown in Table 1. Among the quinolone compounds, the inhibitory activity of DU-6859a was the highest, followed by DV-7751a, CPFX, DR-3355, SPFX, NA, and DR-3354, in that order. Novobiocin was also tested for its inhibition of topoisomerase IV decatenation. The $IC_{50}$ of novobiocin was similar to those seen with the fluoroquinolones. The correlation between the $IC_{50}$s for decatenation of topoisomerase IV and those for supercoiling of DNA gyrase from *E. coli* KL-16 is shown in Fig. 5. The inhibitory activities for quinolones against topoisomerase IV decatenation correlated well with the inhibitory activities against DNA gyrase supercoiling. The correlation coefficient was calculated as 0.658 for all quinolones or 0.918 when data for DR-3354 and NA were omitted.

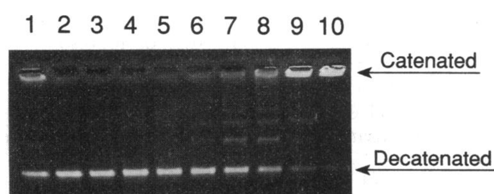


FIG. 3. Effects of potassium ion on topoisomerase IV-catalyzed decatenation. Standard reaction mixtures containing 50, 60, 70, 80, 90, 100, 110, 120, 130, or 140 mM KCl (lanes 1 to 10, respectively) were incubated for 15 min.

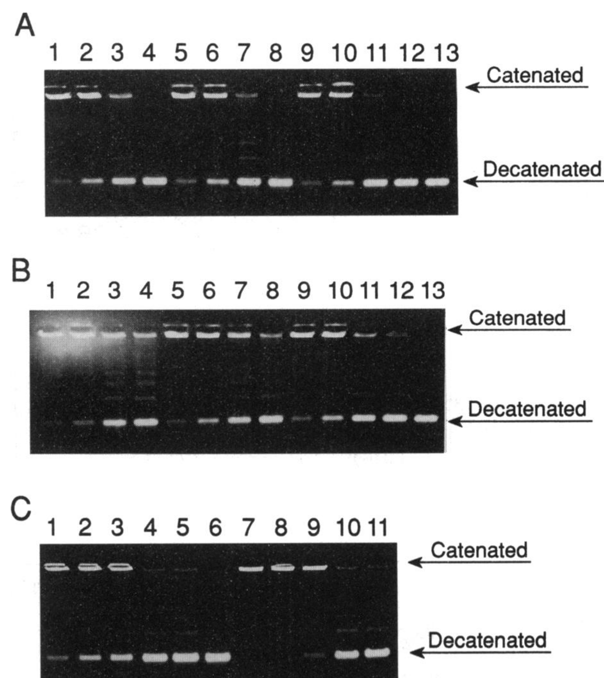


FIG. 4. Inhibitory activities of quinolones and novobiocin against the decatenation reaction of topoisomerase IV. (A) Lanes 1 to 4, 3.13, 1.56, 0.78, and 0.39 μg of DU-6859a per ml, respectively; lanes 5 to 8, 6.25, 3.13, 1.56, and 0.78 μg of DV-7751a per ml, respectively; lanes 9 to 12, 12.5, 6.25, 3.13, and 1.56 μg of DR-3355 per ml, respectively; lane 13, no inhibitor. (B) Lanes 1 to 4, 800, 400, 200, and 100 μg of DR-3354 per ml, respectively; lanes 5 to 8, 12.5, 6.25, 3.13, and 1.56 μg of CPFX per ml, respectively; lanes 9 to 12, 12.5, 6.25, 3.13, and 1.56 μg of SPFX per ml, respectively; lane 13, no inhibitor. (C) Lanes 1 to 5, 400, 200, 100, 50, and 25 μg of NA per ml, respectively; lane 6, no inhibitor; lanes 7 to 11, 25, 12.5, 6.25, 3.13, and 1.56 μg of novobiocin per ml, respectively.

TABLE 1. Inhibitory activities of quinolones and novobiocin against topoisomerase IV decatenation and DNA gyrase supercoiling

| Antibacterial agent | $IC_{50}$ (μg/ml) for: | | A/B ratio |
|---|---|---|---|
| | Topoisomerase IV decatenation[a] (A) | DNA gyrase supercoiling[b] (B) | |
| DU-6859a | 1.47 | 0.082 | 17.93 |
| DV-7751a | 3.22 | 0.21 | 15.33 |
| DR-3355 | 5.95 | 0.38 | 15.66 |
| DR-3354 | 257 | 4.7 | 54.68 |
| CPFX | 5.7 | 0.21 | 27.14 |
| SPFX | 8.27 | 0.47 | 17.60 |
| NA | 187 | 23 | 8.13 |
| Novobiocin | 4.78 | NT | |

[a] Assays were performed as described in Materials and Methods.
[b] Data from previous studies (12, 13). NT, not tested.

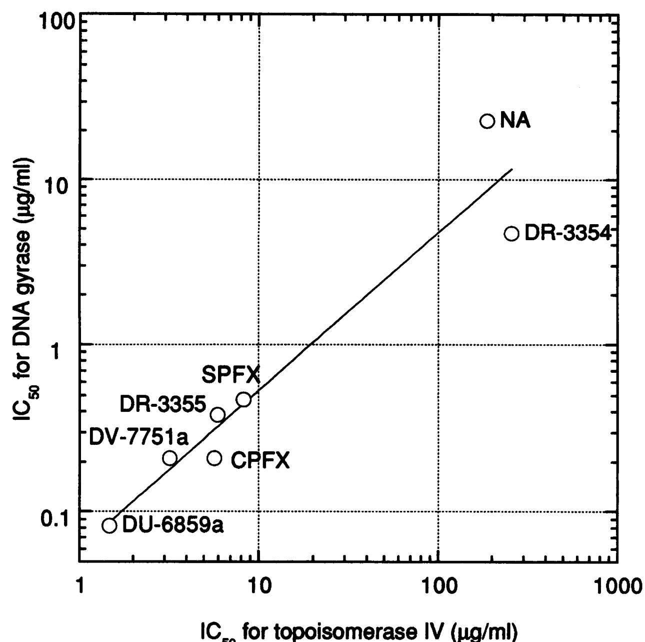


FIG. 5. Correlation between inhibition of topoisomerase IV and that of DNA gyrase (data from previous study [13]). Correlation coefficient = 0.658.

## DISCUSSION

In this study, we assessed the inhibitory potencies of quinolone compounds against topoisomerase IV in order to clarify the action of these drugs against *E. coli* topoisomerases. Topoisomerase IV has two enzymatic activities: decatenation and relaxation (18, 27). The most likely function of topoisomerase IV in the bacterial cell is the segregation of replicating bacterial chromosomes or plasmids (1, 11, 21, 28, 40). In addition, the specific activities of this enzyme suggest that topoisomerase IV seems to prefer decatenation to relaxation. However, despite these findings, it is still possible that relaxation by topoisomerase IV may occur in bacterial cells under certain conditions.

The quinolones were less active against topoisomerase IV decatenation than against DNA gyrase supercoiling. Nevertheless, quinolone activity was still considerably greater against topoisomerase IV decatenation than against topoisomerase I or topoisomerase III relaxation (2, 25). The $IC_{50}$ for NA against topoisomerase IV decatenation was eight times higher than the $IC_{50}$ for DNA gyrase supercoiling. However, Peng and Marians (27) found only a 2.5-fold difference in the inhibitory activities for NA against topoisomerase IV and DNA gyrase. This inconsistency may have occurred because in that study relaxation by topoisomerase IV or DNA gyrase was investigated, but in this study decatenation by topoisomerase IV and supercoiling by DNA gyrase was investigated. In addition, different reaction conditions were used to measure the inhibitory activities of quinolones against these type II topoisomerases. Since DNA relaxation by DNA gyrase or topoisomerase IV is not likely to be relevant in vivo, it is better to compare 4-quinolone inhibition against topoisomerase IV decatenation and that against DNA gyrase supercoiling. Peng and Marians (27) also reported that norfloxacin inhibited topoisomerase IV decatenation or relaxation to a similar extent. In our system, we found a fivefold-greater specific activity for topoisomerase IV decatenation than for relaxation. Furthermore, if the same amount of topoisomerase IV was added for both decatenation and relaxation assays, the $IC_{50}$s of some quinolone compounds for relaxation were significantly lower than those for decatenation. For example, the $IC_{50}$s of DR-3355 for topoisomerase IV-catalyzed relaxation and decatenation were 1.8 and 80 µg/ml, respectively (data not shown). Since the decatenation activity of topoisomerase IV seems to be important in vivo, we believe that the decatenation assay is the most relevant way of analyzing inhibitors of topoisomerase IV.

The comparison between the effect of DR-3355 and that of its relatively inactive stereoisomer DR-3354 (10, 12) is especially interesting. As expected, DR-3355 showed lower $IC_{50}$s against DNA gyrase and topoisomerase IV than DR-3354. However, DR-3355 was 43-fold more potent against topoisomerase IV than DR-3354 but only 12-fold more potent against DNA gyrase. This result may perhaps suggest that the difference in potency between these two stereoisomers involves anti-topoisomerase IV activity.

We have found previously that the quinolone MICs for some quinolone-resistant strains which possess highly quinolone-resistant DNA gyrases are not as high as would be expected (13). This phenomenon may be explained, however, if topoisomerase IV is a target for quinolones, because the quinolone $IC_{50}$s against DNA gyrase for quinolone-resistant strains possessing altered DNA gyrases are likely to be in excess of the quinolone $IC_{50}$s against topoisomerase IV. Therefore, in this case, the MIC will be influenced by two factors, inhibition of DNA gyrase and topoisomerase IV. However, $IC_{50}$s for quinolones against topoisomerase IV as well as against DNA gyrase are consistently higher than MICs, which has been an enigma for some time. This anamoly may have been explained by the recent finding that DR-3355 uptake by *E. coli* at low initial inoculum sizes can reach levels over 100 times greater than the external concentration (26). This finding means that quinolones should reach concentrations within bacterial cells high enough to inhibit topoisomerase IV as well as DNA gyrase.

Bacteria possessing *parC* or *parE* genotypes can be phenotypically compensated for by increasing the gene dosage of *gyrA* and *gyrB* genes. However, *gyrA* or *gyrB* mutations cannot be compensated for by increased gene dosage of *parC* and *parE* (15). Recently, Soussy et al. (33) reported that a quinolone-resistant mutant harboring a mutation in the region of *parC* and *parE* (*nfxD*) required an additional mutation in *gyrA* to express the quinolone resistance. These facts reconfirm the theory that the primary target of the quinolones is most likely to be DNA gyrase. Nevertheless, it is possible that topoisomerase IV is a secondary target for the quinolones, especially for bacteria that contain a quinolone-resistant DNA gyrase. This may show the potential of topoisomerase IV as a target for new antibacterial agents.

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