Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11801–11805, December 1995
Biochemistry

Topoisomerase IV is a target of quinolones in *Escherichia coli*

(DNA gyrase/DNA replication/catenanes/norfloxacin/drug resistance)

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ABSTRACT **We have demonstrated that, in *Escherichia coli*, quinolone antimicrobial agents target topoisomerase IV (topo IV). The inhibition of topo IV becomes apparent only when gyrase is mutated to quinolone resistance. In such mutants, these antibiotics caused accumulation of replication catenanes, which is diagnostic of a loss of topo IV activity. Mutant forms of topo IV provided an additional 10-fold resistance to quinolones and prevented drug-induced catenane accumulation. Drug inhibition of topo IV differs from that of gyrase. (*i*) Wild-type topo IV is not dominant over the resistant allele. (*ii*) Inhibition of topo IV leads to only a slow stop in replication. (*iii*) Inhibition of topo IV is primarily bacteriostatic. These differences may result from topo IV acting behind the replication fork, allowing for repair of drug-induced lesions. We suggest that this and a slightly higher intrinsic resistance of topo IV make it secondary to gyrase as a quinolone target. Our results imply that the quinolone binding pockets of gyrase and topo IV are similar and that substantial levels of drug resistance require mutations in both enzymes.**

The quinolone antibacterial agents have had a long and important history in the clinic and in basic research. The biological activity of the founding member of the group, nalidixic acid, was discovered in 1965 (1). Successive generations of drugs have brought orders of magnitude increases in efficacy and they are now one of the most widely used classes of antibacterial agents. The primary target of these drugs in *Escherichia coli* was established in 1977 as DNA gyrase, a type-2 topoisomerase (2, 3). The critical role of gyrase is to unlink chromosomal DNA during its replication by the introduction of negative supercoils (4, 5). The quinolones inhibit gyrase activity *in vitro* and a single amino acid change can cause a 10- to 100-fold decrease in drug sensitivity (6).

The potency of the quinolones is caused by their striking mode of action. It was shown in 1979 that they block DNA synthesis not by depriving the cell of gyrase but by converting gyrase to a poison of replication (7). Anticancer drugs that inhibit human type-2 topoisomerases also convert their targets into poisons (8). The poisoning is mediated by trapping of an intermediate in topoisomerization, which ultimately leads to a double-strand break of DNA (9).

A number of results have implicated secondary targets for quinolones. Chief among these is the multistep resistance to quinolones that occurs clinically, in which mutations in gyrase are only an initial step (10). A second bacterial type-2 topoisomerase was discovered with a sequence similar to that of gyrase, particularly in the region responsible for drug sensitivity (11). This essential enzyme, topoisomerase IV (topo IV), is required for the terminal stages of unlinking of DNA during replication (11, 12). The first evidence that topo IV might be a quinolone target was the demonstration that it is inhibited by quinolones *in vitro* almost as well as gyrase (13). Recently, comparative analyses of DNA sequences have been adduced to imply that topo IV is the primary target of quinolones in *Staphylococcus aureus* and the secondary one in *Neisseria gonorrhea* (14, 15). What has been lacking is a direct demonstration that topo IV is a quinolone target in any organism by showing that drug-resistant mutants encode a resistant topo IV and that a signature activity of topo IV is inhibited *in vivo* by quinolones.

The observation that mutations in topo IV cause accumulation of catenated intermediates in DNA replication provides just such an assay (12). We find that quinolones induce catenane accumulation in *E. coli* if gyrase is first protected from inhibition by mutation. We constructed, and found in a clinically resistant strain, mutations in topo IV that result in resistance to quinolones *in vivo* and with purified enzymes *in vitro*. We also show that the mode of topo IV inhibition is different from that of gyrase, and this difference is a major reason why topo IV is a secondary target for these drugs in *E. coli*.

MATERIALS AND METHODS

Strains, Plasmids, Enzymes, and Assays. Bacterial strains and plasmids are shown in Table 1. The GyrA and GyrB subunits of DNA gyrase were purified to homogeneity as described (18). ParC, ParC^{L80}, ParC^{K84}, and ParE were purified to ≥95% homogeneity (19). Norfloxacin, ciprofloxacin, nalidixic acid, and oxolinic acid were obtained from Sigma; fleroxacin and 2-pyridones were from Abbott. DNA supercoiling by DNA gyrase was assayed as described (20). DNA relaxation and decatenation by topo IV were carried out in 25 mM Tris·HCl, pH 7.6/100 mM potassium glutamate/10 mM $MgCl_2$/0.5 mM dithiothreitol/30 μg of bovine serum albumin per ml/1 mM ATP/120 fmol of pBR322/1.2 units of enzyme [1 unit of activity is the relaxation (or supercoiling) of 120 fmol of pBR322 in 30 min at 37°C]. Reactions were stopped with EDTA, digested with proteinase K, and analyzed on 1% agarose Tris acetate/EDTA (TAE) gels. CC_{50} (50% of maximum DNA cleavage) was determined with relaxed and supercoiled DNA for gyrase and topo IV, respectively, using filter binding (21) and electrophoretic assays.

Assays of Topology, Replication, and Antibiotic Susceptibility. Cells grown in LB or in M9 medium supplemented with 0.2% glucose were exposed to quinolones for 15 min. An equal volume of 75% ethanol/21 mM sodium acetate, pH 4.8/2 mM EDTA/2% phenol was added and plasmid DNA was isolated by alkaline or Triton X-100 lysis. To observe the pattern of catenation, plasmid DNA was nicked by DNase I in the presence of ethidium bromide and resolved on 1% agarose TAE/SDS gels. To determine the extent of supercoiling, DNA was run on a 1% agarose TAE gel containing 10 μg of chloroquine per ml. To analyze newly replicated plasmid DNA, we incubated cells for 15 min in the presence of drug. Then 10 μCi (82 Ci/mmol; 1 Ci = 37 GBq) of [^{3}H]thymidine was added to 1.0 ml of bacterial culture, and after 1 min DNA was

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Abbreviations: topo IV, topoisomerase IV; wt, wild type.

Table 1. Bacterial strains and plasmids

Name	Genotype	Ref. or parent plasmid
Strain		
205096		16
LZ5	C600 *gyrA*^{L83}	Unpublished work
LZ6	C600 *gyrA*$^{+}$	Unpublished work
1596	C600 *gyrA*^{L83} *parC*^{L80}, Kan^{R}	This work*
1597	C600 *gyrA*$^{+}$ *parC*^{L80}, Kan^{R}	This work*
1608	C600 *gyrA*^{L83} *parC*$^{+}$, Kan^{R}	This work*
1609	C600 *gyrA*$^{+}$ *parC*$^{+}$, Kan^{R}	This work*
1643	C600 *gyrA*^{L83} *parC*^{K84}, Kan^{R}	This work*
1644	C600 *gyrA*$^{+}$ *parC*^{K84}, Kan^{R}	This work*
Plasmid		
pCM600	4.72 kb, oriC	12
pRP1	7.3 kb, vector + 1.5-kb *parC*$^{+}$ Kan^{R} + 1.5-kb *parC–parE* intergenic sequence	pBluescript SK(+)
pAW1	6.27 kb, wt *parC*	pACYC184
pAR1	6.27 kb, Leu-80 *parC*	pACYC184
pUW1	7.5 kb, wt *parC*, P1 origin	pEH9
pUR1	7.5 kb, Leu-80 *parC*, P1 origin	pEH9

*Strains were constructed by allelic replacement (17) using pRP1 plasmid carrying either wild-type (wt) or mutant portions of *parC* and subsequent P1 transduction into strain C600.

isolated. DNA synthesis was measured by [^{3}H]thymidine incorporation into trichloroacetic acid-insoluble material.

RESULTS

Inhibition of Purified Topo IV and DNA Gyrase by Quinolones. We assayed eight different quinolones to determine whether any were more effective on purified topo IV than on gyrase. Our hope was that with such a drug we could determine whether topo IV was a target *in vivo* by assaying for accumulated catenated intermediates (12, 28). The drugs included the archetype of the quinolone family, nalidixic acid; the clinically important ciprofloxacin, norfloxacin, and fleroxacin; and representatives of a new generation of drugs, the 2-pyridones (22). Our assays were cleavage of duplex DNA, relaxation and decatenation for topo IV, and supercoiling for gyrase. All of the drugs were ≈2-fold less effective on topo IV than on gyrase, even though the K_i values for the drugs varied by 2 orders of magnitude (Table 2). Therefore, all the drugs tested were inhibitors of topo IV *in vitro*, but none inhibited it preferentially.

Norfloxacin Causes Catenane Accumulation in Drug-Resistant Gyrase Mutants. Given the lack of preferential inhibition of topo IV *in vitro*, we used a genetic means to attenuate quinolone inhibition of gyrase *in vivo*. We used a gyrase mutant (*gyrA*^{r}; S83L) that is 10- to 100-fold more resistant than the wild-type (wt) strain to a range of quinolones (6). We assayed for inhibition of topo IV in the *gyrA*^{r} mutant by accumulation of catenated pBR322 or pCM600. We used three control assays for DNA gyrase function *in vivo*. The first was DNA synthesis. The conversion of gyrase to a replication poison by drugs immediately stops DNA synthesis (1), whereas topo IV is not required for synthesis (12). The second was DNA supercoiling. Gyrase is uniquely required for the introduction of negative supercoils, whereas topo IV has no appreciable role in the maintenance of supercoiling (12). The third is DNA knotting, which increases upon inhibition of gyrase (23) but not of topo IV (12).

Table 2. Summary of topo IV and gyrase inhibition by quinolones and 2-pyridones

	Topo IV		DNA gyrase	
	K_i, μM	CC_{50}, μM	K_i, μM	CC_{50}, μM
Nalidixic acid	9.8	ND	8.3	ND
Fleroxacin	4.0	4.2	1.7	2.0
Norfloxacin	2.1	2.0	0.9	0.9
2-Pyridone-B7	1.8	1.7	0.5	0.9
2-Pyridone-A6	1.6	3.0	1.4	2.1
Ciprofloxacin	0.5	0.5	0.2	0.3
2-Pyridone-B4	0.4	0.6	0.2	0.3
2-Pyridone-B1	0.2	0.3	0.1	0.3

ND, not determined.

Fig. 1*A* shows that DNA synthesis was reduced by two-thirds at 0.4 and 50 μM norfloxacin for *gyrA*$^{+}$ and *gyrA*^{r} strains, respectively. This sets the range over which we could assay for inhibition of topo IV in the *gyrA*^{r} mutant. Higher concentrations would block DNA synthesis even in the mutant and thereby preclude replication catenane accumulation. If lower drug concentrations preferentially inhibited topo IV, catenane accumulation would have already been observed in wt strains and it has not. In the *gyrA*^{r} mutant, addition of norfloxacin

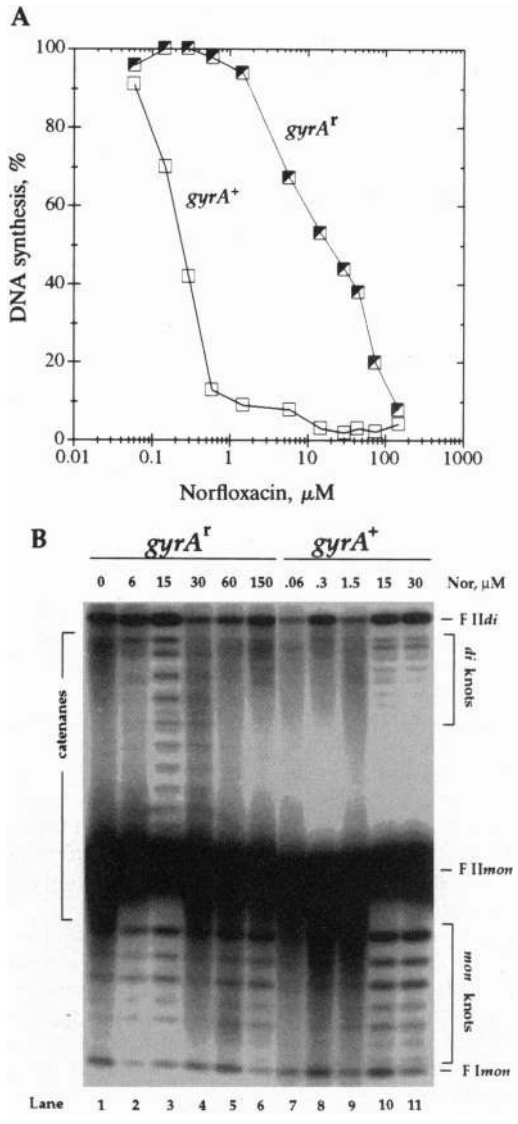

FIG. 1. (*A*) Norfloxacin inhibition of [^{3}H]thymidine incorporation into DNA in *gyrA*^{r} (◩) and *gyrA*$^{+}$ (□) strains. Cells were treated with drug for 15 min prior to the 1-min pulse with [^{3}H]thymidine. (*B*) Effect of norfloxacin on catenane accumulation. *gyrA*^{r} (lanes 1–6) or *gyrA*$^{+}$ (lanes 7–11) strains were incubated with norfloxacin for 15 min at 37°C. Plasmid DNA was isolated and analyzed by agarose gel electrophoresis. Positions of supercoiled (F I), nicked (F II) monomer (*mon*) or dimer (*di*) rings, knots, and catenanes are shown.

gave the ladder and amounts of catenated replication intermediates characteristic for topo IV inhibition (Fig. 1*B*). Similar results were obtained with plasmid pCM600 (data not shown). The optimal concentrations of norfloxacin for catenane accumulation were 15–30 μM. These catenanes must derive from replication because they have the characteristic electrophoretic pattern and are absent at drug doses that block DNA synthesis (lanes 5 and 6).

Norfloxacin did not cause catenane accumulation in *gyrA*$^+$ strains, where it instead blocked DNA replication (Fig. 1*B*). We confirmed by two additional assays that gyrase was not inhibited by norfloxacin, at the levels used, in the *gyrA*^{r} strain. First, knotting was seen at drug concentrations 10-fold higher than catenanes were (Fig. 1*B*). Second, the supercoiling of plasmid pBR322 was unchanged in the *gyrA*^{r} strains at norfloxacin concentrations that induced catenation of the same plasmid (data not shown). An effect on supercoiling was not seen until drug levels exceeded 60 μM in the *gyrA*^{r} strain.

Quinolones Cause High Levels of Catenane Accumulation of Newly Synthesized DNA. The relatively low levels of catenanes seen in Fig. 1*B* and in topo IV mutants at the nonpermissive temperature represent the steady-state amount (12). Pulse labeling with [^{3}H]thymidine showed that nearly all of the newly replicated plasmids in topo IV mutants proceeded through catenated intermediates (28). Therefore, we analyzed the effect of quinolones on newly replicated plasmid DNA in *gyrA*^{r} cells. Cells were labeled for 1 min with [^{3}H]thymidine after a 15-min preincubation with 15 μM norfloxacin. The plasmid DNA was isolated, nicked, and resolved by agarose gel electrophoresis. Half of the labeled plasmid DNA was catenated, ranging in complexity from 2 to 68 nodes (Fig. 2*A*), the same profile as found in topo IV mutants after a 1-min pulse (28). Plasmid supercoiling, and thus gyrase activity, was not affected by the drug (Fig. 2*B*). Although we used norfloxacin in most of our experiments, other quinolones also inhibited topo IV in the *gyrA*^{r} strain. For a 1-min pulse, 40 μM ciprofloxacin or 27 μM fleroxacin led to incorporation of one-half of the label into catenanes.

A Mutation in *parC* Confers Quinolone Resistance *in Vitro*. The single amino acid change in gyrase that causes the greatest resistance to quinolones is S83L in GyrA (24), the mutation used in the above experiments. We constructed the analogous mutation in topo IV by mutating the AGC codon of Ser-80 in *parC* to CTC, which encodes Leu. This mutant ParC (ParC^{L80}) was overexpressed and purified to homogeneity. Enzymatic activity was reconstituted by adding purified ParE to either ParC^{L80} or ParC$^+$. The enzymes had similar specific activities—2 × 10^6 relaxation units per mg of protein. Relaxation was 50% inhibited at 7 μM with wt topo IV but required 75 μM norfloxacin with the mutant enzyme (Fig. 3*A*). Similar results were obtained for norfloxacin-induced cleavage (Fig. 3*B*). The CC_{50} for topo IV^{L80} was 62 μM compared to 6 μM for topo IV$^+$ (Fig. 3*B*). Inhibition of decatenation gave the same relative difference between the ParC^{L80}- and ParC$^+$-containing enzymes. Thus, the S80L mutation causes a 10-fold increase in resistance to norfloxacin for topo IV, roughly the same increase in resistance as Ser-83 to Leu causes in gyrase.

ParC^{L80} Confers Quinolone Resistance to a GyrA^r Strain. To determine whether ParC^{L80} confers resistance to norfloxacin *in vivo*, we constructed four isogenic strains that carry the *gyrA*$^+$ or *gyrA*^{r} allele and the *parC*$^+$ or *parC*^{L80} gene in all pairwise combinations. The single colony survival results are shown in Fig. 4. The concentration of norfloxacin that inhibited growth by one-half for the *parC*^{L80} *gyrA*^{r} strain was ≈6 times higher than that for the *parC*$^+$ *gyrA*^{r} mutant. The *parC*^{L80} mutation did not confer any drug resistance to the *gyrA*$^+$-containing strains. Similar results were obtained with ciprofloxacin and fleroxacin. These results establish that topo IV is a target of quinolones *in vivo*.

We used the single (*gyrA*^{r}) and double (*parC*^{L80} *gyrA*^{r}) drug-resistant mutants to test whether inhibition of topo IV was solely responsible for accumulation of replication catenanes. If topo IV were indeed the drug target in the *gyrA*^{r} strain, then catenanes should no longer accumulate when topo IV is made drug resistant. This is the result we obtained (Fig. 2*C*). Thus, the change of Ser-80 to Leu renders topo IV drug resistant *in vivo* and *in vitro*.

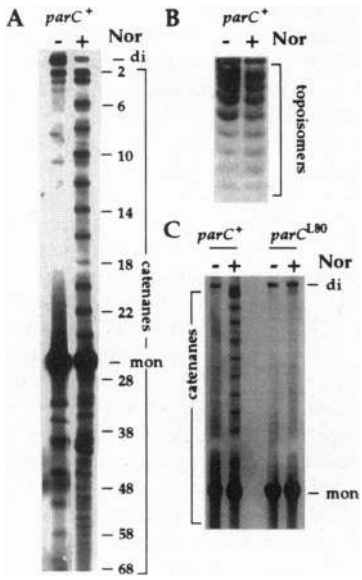

FIG. 2. Effect of norfloxacin on newly replicated plasmid DNA in *gyrA*^{r} strains. Cells were incubated with either 15 μM norfloxacin (lanes +) or no drug (lanes −) and treated as described in Fig. 1*B*. Shown are autoradiographs of the agarose gels. Positions of monomer (mon) and dimer (di) plasmid are indicated. All strains are *gyrA*^{r}. (*A*) Catenane accumulation. (*B*) DNA supercoiling. (*C*) Catenane accumulation in *parC*$^+$ or *parC*^{L80} strains.

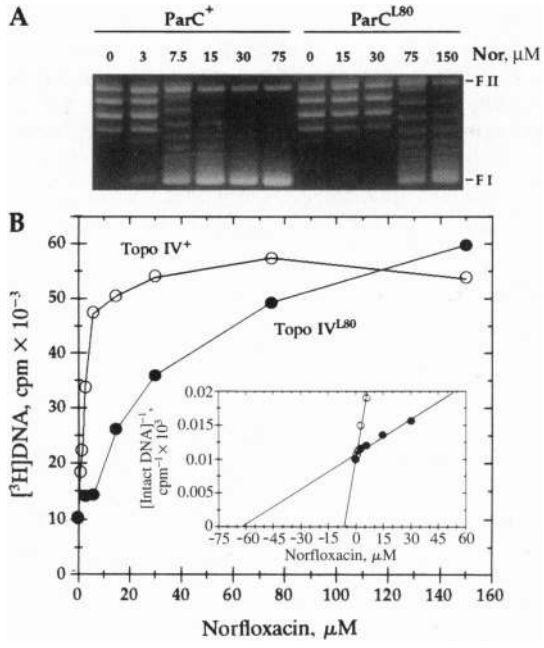

FIG. 3. Norfloxacin susceptibility of purified wt and ParC^{L80} topo IV. (*A*) Drug inhibition of topo IV relaxation activity. Reaction mixtures contained either ParC$^+$ or ParC^{L80} reconstituted with excess ParE. Norfloxacin concentrations are indicated above the ethidium bromide-stained gel. (*B*) Induction of topo IV cleavage by norfloxacin. Topo IV$^+$ (○) or topo IV^{L80} (●) was reacted with ^{3}H-labeled supercoiled plasmid. The enzyme-attached cleaved DNA bound to filters was measured. The average of three experiments is shown. (*Inset*) Semireciprocal plot.

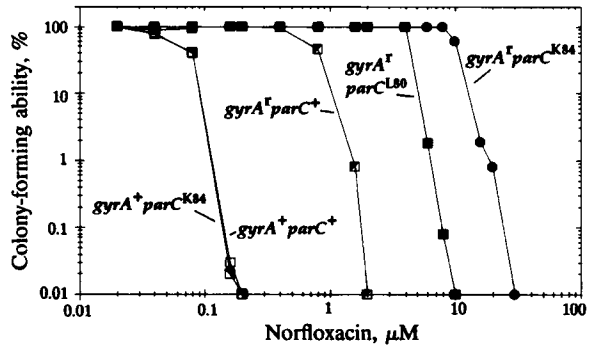

FIG. 4. Norfloxacin inhibition of colony formation. Mutants of C600 were grown to midlogarithmic phase and plated on LB plates supplemented with norfloxacin. The number of colonies was normalized to that without drug.

A Quinolone-Resistant Clinical Isolate Has a Mutant Topo IV. Does a drug-resistant mutation in *parC* also occur in nature? A clinical strain, 205096, has been isolated that contains several mutations that collectively confer 1000-fold resistance to quinolones (16). This strain has mutations in Ser-83 and Asp-87 of GyrA, and we predicted that topo IV was also mutated. To test this, we sequenced 351 nucleotides of *parC* in strain 205096. We found only 1 nucleotide change leading to an E84K mutation. Mutations in the homologous residue in DNA gyrase confer drug resistance, although not as well as the S83L mutation (24). The purified mutant ParC^{K84} plus ParE was 25-fold more resistant to norfloxacin than the reconstituted enzyme containing ParC^{+}. We constructed the test strains identical to those used above and found *in vivo* that the naturally occurring mutation in *parC* caused 10-fold more resistance than *gyrA*^{r} alone (Fig. 4)—i.e., twice that of *parC*^{L80}.

Inhibition of Topo IV Slowly Arrests DNA Replication: Dominance Tests of the Resistant Alleles. When *gyrA*^{r} *parC*$^{+}$ cells were incubated with norfloxacin at concentrations just high enough to prevent growth, we observed a gradual inhibition of the rate of [^{3}H]thymidine incorporation (Fig. 5*A*). This contrasts with the rapid inhibition of DNA replication in *gyrA*$^{+}$ strains (Fig. 5*A*). This characteristic rapid block in replication results because the drug converts gyrase into a potent poison of DNA replication (7).

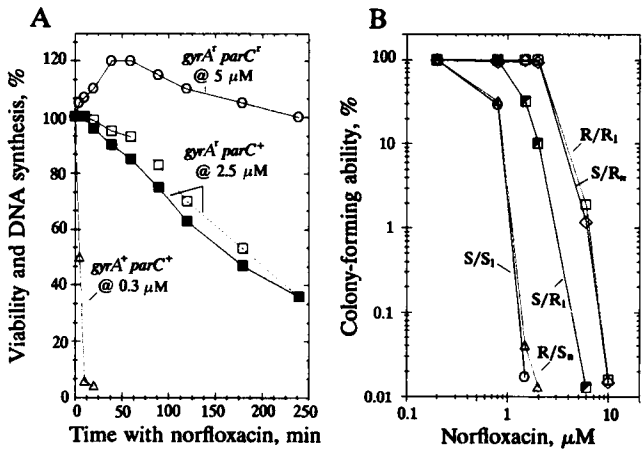

FIG. 5. Cytotoxic effect of norfloxacin. (*A*) Time course of the effect of norfloxacin on cell viability (solid symbols) and [^{3}H]thymidine incorporation (open symbols). Viability was determined as the number of colonies on LB plates after the indicated times of incubation with drug in liquid medium. (*B*) Effect of norfloxacin on colony-forming ability of strains containing both resistant and wt ParC. The number of colonies formed on LB plates supplemented with norfloxacin after 14 hr of incubation at 37°C is plotted. R, resistant allele; S, wt allele. Subscript refers to copy number of the plasmid-borne allele; n = 5–15.

Removal of gyrase activity by mutation causes a much slower diminution of DNA synthesis (7).

Another manifestation of the poison effect is the dominance of *gyrA*$^{+}$ over *gyrA*^{r} (25). Eukaryotic type-2 topoisomerases are also made into poisons by anticancer agents and the wt allele is also dominant over the resistant allele (26). For catalytic inhibitors, which act by depriving the cell of their targets, the resistant allele should be dominant. To test dominance for topo IV, we constructed all combinations with *parC*$^{+}$ or *parC*^{r} alleles expressed from the chromosome and from plasmids, either single copy or multicopy. We measured the effect of norfloxacin on colony formation (Fig. 5*B*). With *parC* on a single copy plasmid and the chromosome, the heterozygote is midway in sensitivity between the homozygous wt and resistant strains—i.e., codominance. Multicopy plasmids do not alter the phenotype of homozygous strains. However, the multicopy allele (whether *parC*$^{+}$ or *parC*^{r}) dominates for the heterozygotes. Therefore, the dominance tests give results different from that obtained with either classical topoisomerase poisons or catalytic inhibitors.

DISCUSSION

Topo IV Is a Quinolone Target *in Vivo*. We have established that topo IV is a target for quinolones in *E. coli*. This was not apparent in earlier studies of wt strains because bactericidal antigyrase concentrations of drug were lower than that needed to target topo IV. Even at drug concentrations that inhibited both topoisomerases, the gyrase effect was epistatic because of an immediate poisoning of DNA replication. Two results establish that topo IV is a quinolone target. First, in a *gyrA*^{r} strain, these drugs caused accumulation of replication catenanes and cell death without a significant effect on DNA replication (Figs. 1 and 4). The fraction of DNA that is catenated and the catenane node distribution (Fig. 2*A*) were identical to those seen in topo IV mutants (12, 28). Second, we constructed two mutant forms of ParC that conferred 5- or 10-fold additional quinolone resistance to a *gyrA*^{r} strain (Fig. 4). Topo IV reconstituted from the purified mutant forms of ParC was 10- and 25-fold more drug resistant than wt enzyme (Fig. 3). Moreover, quinolones did not cause catenane accumulation in the gyrase and topo IV drug-resistant double mutant strain (Fig. 2*C*).

Additional evidence indicates that topo IV is a quinolone target *in vivo*. First, a single mutation in *gyrA* of *E. coli* leads to a 100-fold resistance to quinolones as measured by the arrest of DNA synthesis (Fig. 1*A*). At the same time, resistance measured by colony-forming ability goes up only 10-fold (Fig. 4). The discrepancy is due to topo IV, because *gyrA*^{r} mutants that are also *parC*^{r} are an additional 10-fold more resistant (Fig. 4). Second, sequence analysis of genes that are homologous to *E. coli parC* led to the conclusion that mutations in *parC* can confer quinolone resistance to *S. aureus* (14) and *N. gonorrhea* (15). *S. aureus* has, presumably, a naturally drug-resistant gyrase (27), and S80F or S80Y mutations in the ParC homolog gave 10- to 15-fold resistance. In this organism, therefore, topo IV seems to be the primary target for quinolones. In the *N. gonorrhea* study, topo IV was concluded to be a secondary target. The change S88F in the *parC* homolog caused 8-fold resistance to quinolones but only if the strain already had a gyrase-resistance mutation. Mutation E91K provided an additional 4-fold resistance. Thus, in both species, quinolone-resistant mutations in *parC* occurred in positions homologous to ones that we demonstrated confer drug resistance to *E. coli*.

Why Topo IV Is Secondary to Gyrase as a Quinolone Target. Why is DNA gyrase, and not topo IV, the primary quinolone target in *E. coli*? The possible explanations include (*i*) the intrinsic sensitivity of topo IV to quinolones is less than that of gyrase; (*ii*) the *in vivo* conditions selectively diminish the inhibition of topo IV; and (*iii*) the modes of action of the

enzymes translate into less efficient drug inhibition of topo IV. We believe that possibility *iii* is the major reason why topo IV is secondary to gyrase as a quinolone target, but that *i* makes a contribution as well.

We tested the first possibility by studying the relative sensitivities of purified DNA gyrase and topo IV to a variety of quinolone drugs *in vitro* (Table 2). For all the drugs, gyrase was only 2-fold more sensitive than topo IV. This difference cannot explain the 10-fold increase in minimal inhibitory concentration resulting from a drug-resistant mutation in gyrase. We conclude that although topo IV is intrinsically slightly less sensitive to quinolones than gyrase, this is not the major reason why it is a secondary target. In considering the second possibility, we note that supercoiling (gyrase) and decatenation (topo IV) were inhibited by 50%, respectively, at 6 and 15 μM norfloxacin *in vivo* (Fig. 1; data not shown). We conclude that topo IV is only 2–3 times less sensitive to quinolones than DNA gyrase *in vivo*.

We now turn to possibility *iii*. The primary effect of quinolones on bacteria is instant arrest of DNA replication at drug concentrations that kill the cell (1). A single mutation in gyrase rescues DNA replication and viability (6, 25). Bactericidal drug concentrations are 100 times lower than concentrations that inhibit gyrase supercoiling activity *in vivo* because quinolones convert gyrase to a poison (7). Because the *gyrA*^{r} strain we used is 10 times more resistant to quinolones than wt cells, and *parC*^{r} introduces another 10-fold resistance, the targeting of topo IV seems to occur at 10 times higher drug concentrations than the targeting of gyrase. This contrasts with *in vitro* and *in vivo* results that topo IV is only 2–3 times less sensitive to norfloxacin than DNA gyrase.

If a target topoisomerase acts ahead of and close to the replication fork, then cleaved adducts will rapidly block replication. If a topoisomerase acts behind the fork, we expect replication to die out slowly. The rapid replication arrest at drug concentrations that affect only gyrase suggests that gyrase acts in front of the fork. Topo IV inhibition by drug (in a *gyrA*^{r} strain) results in a gradual decrease in replication that coincides with cell death (Fig. 5*A*). We suggest that this results from topo IV acting behind the fork and that the presence of an intact sister chromosome allows repair of the double-strand break. Thus, the conversion of the topo IV adduct to a lethal lesion is less likely. We speculate that this explains the slower rate and higher dose for topo IV-mediated killing. This model can also explain the dominance results. When ParC^r and ParC$^+$ were roughly equal, an intermediate level of resistance resulted (Fig. 5*B*). With multicopy plasmids, the allele on the plasmid dominated. We suggest that the resistant and wt ParC compete for DNA and the probability of death is proportional to the number of lesions. Thus, these drugs may convert topo IV to a poison just as readily as they do gyrase, but killing is limited because the poison is slower acting and lesion repair provides an antidote.

Quinolone inhibition of topo IV is primarily bacteriostatic, as shown by the dramatic drop in colony-forming ability at drug concentrations that had a modest effect on viability (Figs. 4 and 5*A*). After addition of drug, cells undergo 1–2 doublings and then cease to divide. These results can be explained by a block in cell division after the first replication cycle due either to an inability to segregate replicated chromosomes when topo IV is inhibited or to lesion accumulation during the first round of replication.

Clinical Implications. We showed that a naturally occurring *E. coli* mutation (E84K) in ParC makes the reconstituted topo IV 25-fold more resistant to quinolones than the wt enzyme. The E84K mutation is more likely to occur *in vivo* than the S80L mutation, because it requires only 1 nucleotide change instead of 2. The *parC*^{K84} and *gyrA*^{r} strain was 10-fold more resistant to drugs than *parC*$^+$ *gyrA*^{r} and an additional 2-fold more resistant than *parC*^{L80} *gyrA*^{r}. Although quinolones have been developed to inhibit DNA gyrase, it is striking that successive generations of drugs giving orders of magnitude increases in potency have increased the action on topo IV in parallel. The drug binding pockets of the two enzymes must have fundamental similarities. Topo IV has unwittingly become a clinically important drug target because of the widespread drug-resistance mutations in gyrase.

We thank D. Bramhill, P. Heisig, C. G. Lerner, K. J. Marians, A. Maxwell, S. Nakamura, G. Plunkett III, and L. L. Shen for materials and assistance. This work was supported by National Institutes of Health Grant GM31657 and National Institute on Environmental Health Sciences Grant ESO1896. E.L.Z. is a Special Fellow of the Leukemia Society of America.

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