The Mechanism of Inhibition of Topoisomerase IV by Quinolone Antibacterials*

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Topoisomerase IV (Topo IV) is a mediator of quinolone toxicity in bacteria. In this work, we demonstrate that norfloxacin, a model quinolone, converts Escherichia coli Topo IV into a poisonous adduct on DNA as opposed to inhibiting topoisomerase activity. Norfloxacin inhibition of Topo IV induces a slow decline in DNA synthesis that parallels cell death. Treatment of cells with a lethal concentration of the antibiotic did not block chromosome segregation, the phenotype of catalytic inhibition of Topo IV. Instead, norfloxacin causes DNA damage, as evidenced by the induction of the SOS pathway for DNA repair; the increase in susceptibility to the drug by mutations in genes for DNA repair pathways including recA, recB, and uvrD; and the efficient detergent-induced linearization of plasmid DNA in drug-treated cells. Wild-type and drug-resistant alleles of Topo IV are co-dominant, but we find that mutations in recA, seqA, or gyrB result in unconditional dominance of the sensitive allele, the characteristic of a poisoning mode of inhibition. These mutations either compromise chromosome integrity or force Topo IV to play a more active role in DNA unlinking in front of the replication fork. We interpret our results in terms of distinct but complementary roles of Topo IV and gyrase in DNA replication.

The quinolone family of antimicrobial agents has contributed to research and therapy for more than 30 years. The patriarchs of this drug family, nalidixic and oxolinic acids, were used to map one of the first drug resistance genetic determinants in Escherichia coli (1) and were among the first antibacterial agents that efficiently inhibited DNA replication (2). In addition, they were important in determining the mechanism and physiological role of type-2 topoisomerases (3, 4). The modern successors of nalidixic and oxolinic acids, fluoroquinolones, are among the most widely used antibacterials in the world today (5). In our research, we focused on norfloxacin, a fluoroquinolone that is commonly used in the laboratory and clinic.

Recently, it has been shown that quinolones target two type-2 DNA topoisomerases, DNA gyrase and topoisomerase IV, in both Gram-negative and Gram-positive bacteria (6–11). Both enzymes are essential and are conserved throughout the eubacterial kingdom (12). Topo IV unlink newly replicated DNA, thereby allowing proper chromosome and plasmid segregation (13, 14). As a result, Topo IV mutants at the nonpermissive temperature accumulate catenated but otherwise complete products of replication. Gyrase is absolutely essential for maintaining negative supercoiling, and gyrase mutants have a variety of effects on plasmid DNA topology and DNA metabolism.

Quinolones completely shut down DNA synthesis within minutes at their minimal effective concentration. One of the quinolone targets, DNA gyrase, is required for DNA replication. Therefore, the simplest hypothesis for quinolone action is that inhibition of DNA gyrase activity blocks replication. However, in vivo and in vitro studies with gyrase inhibitors and the analogous inhibitors of eukaryotic topoisomerases revealed that these drugs act by stabilizing a reversible intermediate, the cleavable complex, in which the topoisomerase is covalently attached to DNA (15–18). Such an intermediate can form a physical barrier in front of the replication fork (19). More frequently, though, the reversible topoisomerase-DNA adduct is converted to an irreversible lesion, which in turn can lead to a double-stranded break in DNA (19, 20). This conversion can in principle be caused by any DNA tracking process: replication, transcription, or helicase action. Killing via both gyrase and Topo IV correlates, however, with inhibition of DNA synthesis (8, 21).

The frequency of collisions between the replication fork and topoisomerase-DNA adducts may be rate-limiting in the chain of responses leading to cell death. This frequency depends on the number and location of enzyme-DNA complexes. A single adduct per chromosome can be lethal if it is sufficiently stable. Short lived adducts may have similar effects if they are close to and downstream of the replication fork. The immediate stop of replication when gyrase is targeted by quinolones can be explained within such a framework. This enzyme alleviates topological stress ahead of the replication fork and therefore probably forms a covalent intermediate just ahead of the fork.

All of the early work on quinolones focused on DNA gyrase, but the finding that Topo IV is also a cellular target for quinolones demanded that the mechanism of quinolone action be revisited. The inhibition of E. coli Topo IV by norfloxacin, unlike the targeting of DNA gyrase, did not lead to a rapid block in DNA replication (8). Moreover, in targeting Topo IV, the minimal inhibitory concentration (MIC) in vivo was as much as 100 times the anti-Topo IV Ki, measured in vitro, whereas this ratio was less than 100 for gyrase inhibition. In addition, the wild-type allele of GyrA dominated over a large excess of drug-resistant GyrA (1), while drug-resistant and wild-type alleles encoding the ParC subunit of Topo IV were co-dominant (8).

Thus, the mechanism of Topo IV targeting appeared to be different from that of gyrase. However, we show in this study that the molecular events behind Topo IV inhibition are similar to the targeting of DNA gyrase: norfloxacin converts a Topo IV-DNA complex into a lesion that damages DNA. Norfloxacin

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The abbreviations used are: Topo, topoisomerase; MIC, minimal inhibitory concentration; MPC, maximal permissive concentration.
action on Topo IV at lethal concentrations does not block chromosome segregation but causes a decline (in some mutants as rapid as by targeting gyrase) of DNA synthesis. We therefore interpret the apparent differences between the targeting of gyrase and Topo IV in terms of their distinctive roles in DNA replication.

MATERIALS AND METHODS

Bacteria—The bacterial strains used in this study are listed in Table I.

Plasmids—The plasmids used were described previously (8). Ampicillin-resistant derivatives were constructed by inserting the gene for β-lactamase into a BamHI site within the chloramphenicol acetyltransferase gene of pACYC184. pGBS1 and pGBS2 are pSK-based plasmids containing the gyrA and gyrAW80 genes, respectively, under control of the parC promoter. Wild-type and drug-resistant open reading frames of the gyrA gene were obtained by polymerase chain reaction from pPH3 and pPH483 (22), respectively. We also used the mini-F-based plasmid, pGY5887, containing recA428 (23) as well as its derivative containing the β-lactamase gene inserted in the unique HindIII site.

The activity of topoisomerases encoded by plasmids was confirmed by their ability to complement temperature-sensitive alleles. In addition, the presence of the S83W mutation in gyrA was confirmed by DNA sequencing and by its ability to confer norfloxacin resistance to strain KNK542 at restrictive temperatures.

Measurement of Norfloxacin Resistance—The susceptibility of bacterial strains to norfloxacin was assessed by the inhibition of colony formation. After 12–16 h, colonies were counted on LB or minimal medium agar plates. To characterize strain susceptibility, we chose three measures: MPC50, the maximal permissive concentration of drug, and MIC90, the minimal inhibitory concentration of drug that prevents growth of at least 50% of the bacterial population, and MIC50 and MIC90, the minimal inhibitory concentration of drug that prevented growth of more than 90 and 99% of the population, respectively. Since all three measures are subject to experimental error, we claimed similar sensitivities for two strains only when at least two concentrations gave identical results in three independent experiments.

In addition to the colony formation assay, the values of the MPC and MICs were confirmed by replica plating from plates with no drug onto plates with antibiotic agent. We used the composite index, MIC = (MPC50 + MIC90 + MIC50)/3, as a measure of strain susceptibility.

Assays of DNA Replication and Topology—E. coli C600 leu thr strains were grown to logarithmic phase in M9 minimal medium. DNA synthesis was measured by [3H]thymidine incorporation into trichloroacetic acid-insoluble material. Incorporation in pulses was stopped by addition of 75% EtOH/21 mM sodium acetate, pH 4.8, 2 mM EDTA, 2% acetic acid-insoluble material. Incorporation in pulses was stopped by addition of 75% EtOH/21 mM sodium acetate, pH 4.8, 2 mM EDTA, 2% acetic acid-insoluble material. Incorporation in pulses was stopped by addition of 75% EtOH/21 mM sodium acetate, pH 4.8, 2 mM EDTA, 2% acetic acid-insoluble material.

DNA synthesis was also measured in synchronized populations. Cells growing in minimal medium were collected at A600 = 0.2 by centrifugation and washed twice with blocking medium: M9 plus 0.5 mM α-methyl-DL-methionine. Cells were incubated in this blocking medium with aeration for 45 min to complete ongoing DNA replication. [3H]thymidine was added for 1 min, and the amount of incorporated [3H]thymidine was normalized to the A600. Background retention of the label was determined as the amount of acid-precipitable counts obtained from cells pretreated and pulsed in the presence of 100 mM hydroxyurea. These background values were subtracted from the experimental points.

Plasmid DNA replication was measured as the amount of [3H]thymidine incorporated into all forms of plasmid DNA after a 1-min pulse. Replication was aborted by the addition of the stop solution described above, and plasmid DNA was isolated according to the standard alkaline lysis procedure (24). The DNA was resolved by electrophoresis through a 1% agarose gel (8) and was visualized by autoradiography following tritium enhancement.

For determination of the supercoiling of the plasmid population, a portion of the plasmid DNA isolated as above was resolved by electrophoresis through a 1% agarose gel containing 10 mg/ml chloroquine in a TAE buffer. The DNA was visualized by Southern blot hybridization. To determine the fraction of plasmid DNA in a cleavable complex, the cells exposed to quinolone were treated with lysozyme and incubated with 1% SDS for 30 min at 37 °C. DNA was then extracted by the standard alkaline procedure and analyzed by electrophoresis through a 1% agarose gel.

Assay of SOS Induction by Norfloxacin—To measure SOS activation by norfloxacin, we measured β-galactosidase activity transcribed from the DNA damage-sensitive sulA promoter. DM4000 derivatives containing a sulA:β-gal fusion as a stable chromosomal insertion (Table I) were grown in medium A (25). When the cell density reached 0.2 at A600, norfloxacin was added. After a 60-min incubation with drug, the A600 was determined, and the assay for β-galactosidase activity was performed at 28 °C for 10 min (25). Two averages of five independent experiments with less than 10% S.D. are presented under “Results.”

Flow Cytometry—Flow cytometry was used to determine the DNA content in a statistically significant number of bacterial cells. Bacteria were grown in LB in the presence or absence of norfloxacin, and 1 ml portions were fixed with 70% ETOH. The cells were harvested by centrifugation and resuspended in 10 mM Tris, pH 7.2, 1.5 mM MgCl2, 150 mM NaCl. A portion of these cells was diluted to approximately 107/ml in a total volume of 1.0 ml and stained with either 0.3 mM Hoechst 33258 or 0.5 mM DAPI (Calbiochem). For convenience, bacterial cells were then stored in the stain solution and shielded from light for 24–36 h at 4 °C. DAPI-stained cells were used to confirm cell count and DNA staining by fluorescence microscopy. Both DAPI- and H33258-stained cells were analyzed using a Coulter Epics Elite flow cytometer equipped with dual UV lasers. H33258 and DAPI were excited at a wavelength of 351 nm, 200-milliwatt power. A 480-nm-long path interference filter (Omega Optica) was used to filter the emitted fluorescence of the dye. A total of 20,000 cells were analyzed in each sample at a rate of 700–800 cells × s−1 through a 60-mm nozzle. The numerical output from three independent experiments was averaged and plotted using Deltagraph Professional.

RESULTS

Norfloxacin Killing of Bacteria through Its Action on Topo IV Is Not Associated with Failure to Segregate Chromosomes—Topo IV can be made the sole important target of quinolones by using gyrA strains to remove the complicating effects of inhibition of gyrase (8). In the gyrA mutants, 0.2–1 μg/ml norfloxacin prevented colony formation and caused cell death. Higher concentrations of drug, 5–15 μg/ml, were needed to block Topo IV activity as measured by the accumulation of replication catenanes in plasmid DNA. Both the loss of viability at low drug concentrations and the accumulation of replication catenanes at higher concentrations were blocked by mutations in the parC gene of Topo IV that confer drug resistance. Thus, the inhibition of Topo IV activity at higher drug concentrations can cause a DNA segregation defect, but the killing of the cells occurs at lower drug concentrations, where most of the enzyme appears to be unaffected.

We studied directly the effect of norfloxacin on bacterial cells opposed to plasmid chromosome replication using flow cytometric analysis of the DNA content of E. coli strains in which either Topo IV or gyrase was targeted. If the loss of viability resulting from Topo IV targeting at low drug concentrations is due to inhibition of Topo IV enzymatic activity, then unsegregated chromosomes should accumulate in cells. This increase in DNA content is readily detected by flow cytometry.

Such a shift in DNA distribution was observed when Topo IV was inhibited by placing parE mutants at a nonpermissive temperature (Fig. 1, compare A and B). However, when either gyrase (D) or Topo IV (F) was targeted by norfloxacin at their respective MICs, the DNA content was unchanged from that of the untreated controls (C and E). In these experiments, the amount of drug and time of exposure was adjusted so that viability was reduced by 95%, the same viability as in the parE mutant at the nonpermissive temperature. Drug-resistant alleles of gyrA and parC (G and H) rescued cell viability for the gyrase and Topo IV targeted cells, which confirms the specificity of the treatments. Indeed, the DNA content in either drug-susceptible strain (D and F) was the same as in the doubly drug-resistant gyrA parC strain (H).

We conclude that norfloxacin killing via targeting Topo IV is not a result of blocking chromosome segregation; Topo IV is still active while the cells are dying.

Targeting Topo IV in Vivo by Norfloxacin Causes SOS Induction—If the antibacterial activity of norfloxacin when targeting
Topo IV is mediated by DNA damage, then it may produce a Topo IV-specific SOS response. SOS induction was examined using the β-galactosidase gene fused to the DNA damage-sensitive promoter of the sulA gene in four isogenic strains carrying either wild-type or drug-resistant alleles of gyrase and Topo IV (Fig. 2). Induction of β-galactosidase was not detected in the doubly resistant strain even at 5 μg/ml norfloxacin. In the other three strains, in which one or both enzymes were inhibited, we observed a nearly linear increase of β-galactosidase with norfloxacin concentration. Inhibition of Topo IV is nearly as effective as inhibition of gyrase in inducing the SOS response.

Previously, the induction of SOS by gyrase targeting was shown to be dependent on RecBCD, presumably because the enzyme generates single-stranded DNA (26, 27). We found that RecBCD is also absolutely necessary for SOS induction due to Topo IV inhibition (Fig. 2). We conclude that the targeting of Topo IV by norfloxacin produces similar DNA damage.

Effect of DNA Damage Repair Mutations on Norfloxacin Sensitivity—So far we established that norfloxacin targeting of Topo IV does not block chromosome segregation but does provoke an SOS response. Both of these observations are consistent with the conversion of a Topo IV-mediated adduct into a DNA damage-induced sulA promoter. The cells contained the β-galactosidase gene under the control of the damage-inducible sulA promoter. The β-galactosidase activity is therefore a measure of SOS induction. Averaged results of five independent experiments are plotted. 

RecA function by a recA deletion (∆recA) caused about an 8-fold drop in drug resistance when either topoisomerase was targeted (Fig. 3). Drug resistance was impaired to a lesser extent, 2–3-fold, in recA428, which is reported to be recombination-deficient but SOS-proficient (23). A similar increase in norfloxacin sensitivity for both targets was observed in the recB21 strain. The lexA and recN mutations had a slightly more pronounced effect on the inhibition of gyrase than of Topo IV (Fig. 3A).

During slow growth (aspartate medium) (Fig. 3B), homologous repair is reduced, because there are only 1–1.2 chromosomes per cell (31). Under these conditions, one would expect that recombination-deficient mutations would have little effect on repair of an adduct. Indeed, no significant effect on either topoisomerase target was detected in five out of six recombination-defective strains tested (Fig. 3B). The exception is the
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To our surprise, a mutation in recA, which had the largest effect on MIC. Despite the paucity of homologous chromosomes, drug sensitivity of a recA strain carrying drug-susceptible gyrase increased almost 10-fold, and the sensitivity of a recA strain in which only Topo IV could be targeted increased 15-fold. Thus, RecA protein seems to have a role in addition to recombination in rescuing bacterial cells from quinolones. In vitro studies indicate that norfloxacin stabilizes a ternary complex with topoisomerase and DNA (32), and such a bulky adduct might be expected to be a target for excision repair. To our surprise, a mutation in uvrA had no detectable effect on the norfloxacin sensitivity of both gyrase- and Topo IV-targeted cells (Fig. 3). Therefore, the major bulky adduct repair pathway is not important for topoisomerase lesion removal.

UvrD encodes a helicase involved in multiple repair pathways. Intriguingly, a uvrD mutation increased bacterial drug sensitivity 4-fold when Topo IV was targeted but had little effect when gyrase was targeted (Fig. 3). When the uvrD mutation was used in combination with the recombination-deficient recA428 mutation, bacteria with drug-susceptible Topo IV growing in rich medium became 10-fold more sensitive to norfloxacin (Fig. 3). Therefore, the major bulky adduct repair pathway is not important for topoisomerase lesion removal.

**Table I**

| Strain | Relevant genotype | Construction or source |
|--------|------------------|------------------------|
| A08110 | recAdel-308 miniF-recA428 | Ref. 23 |
| CAG18501 | recA | Ref. 43 |
| DM4000 | suA:MuA | A. J. Clark |
| GW3703 | uvrD260:Ts5 | Ref. 44 |
| H508 | uvrA | S. Linn |
| HC618 | acrA:Kan | Ref. 45 |
| JCI0298 | recA:Tn10 | A. J. Clark |
| JCI1399 | lexA3 malE3:Ts5 | A. J. Clark |
| LE236 | gyrB21 | Ref. 39 |
| NK063 | seqA:Tet | Ref. 38 |
| RED40 | topA20:Ts10 | Ref. 40 |
| V67 | recB21 | Ref. 27 |

Constructions are shown as donor → recipient, selection. r, resistant; s, sensitive; Tet, tetracycline; Nor, norfloxacin; Kan, kanamycin; Nov, novobiocin; MMC, mitomycin C; Thy, thymidine; Ts, temperature-sensitive; P1 (strain), source of transducing lysate; (1), Tet s revertants of the recA428 strain identified strains were obtained according to Bochner et al. (47); (2), recipients were trimethoprim resistant thyA recA428 (48) was confirmed by polymerase chain reaction.
was not enhanced relative to the single recA428 mutant. As expected, this effect was not detected on aspartate medium (Fig. 3B), because the efficiency of recombination repair is blunted. Because UvrD is a part of postreplicative repair pathways, the results suggest that postreplicative repair is important only for removing Topo IV-DNA adducts. Moreover, these results show that UvrD repairs norfloxacin-mediated Topo IV adducts via a pathway that is different from the UvrABC excision repair pathway.

Effects of Targeting Gyrase and Topo IV on Plasmid DNA Replication—The drug concentration dependence of cleavable complex formation, the precursor of the killing lesion, was measured and compared with the inhibition of DNA replication and of enzymatic activity. The DNA in a cleavable complex is probably already broken, but its continuity is maintained by a topoisomerase bridge. When the enzyme is denatured, the double-stranded break becomes patent, and the enzyme remains covalently attached to each 5'-end. We analyzed plasmid DNA for these studies, because plasmids are more experimentally tractable than the bacterial chromosome. Cells were exposed to a range of norfloxacin concentrations close to the MIC value and pulse-labeled with [3H]thymidine before plasmid DNA was isolated. Three things were measured. 1) To assay cleavable complex formation, the cells were treated with 1% SDS to induce DNA breakage at the site of the complex. 2) After electrophoresis through a 1% agarose gel, the amount of replicated plasmid DNA was determined by tritium enhancement. 3) Electrophoresis through an agarose gel containing chloroquine was used to measure DNA supercoiling.

When gyrA' strains were treated with norfloxacin at concentrations that killed bacteria by targeting Topo IV (0.1–0.5 µg/ml), no inhibition of plasmid DNA replication was detected over the 15-min exposure to a drug (Fig. 4A). 10-Fold higher concentrations caused accumulation of plasmid DNA replication catenanes (8). Therefore, even at concentrations that severely inhibited Topo IV catalysis (2–10 µg/ml), DNA synthesis was not significantly affected, because replication proceeded to the final catenation stage (13). However, norfloxacin at concentrations from 0.1 to 0.5 µg/ml induced a substantial amount of the Topo IV-DNA cleavable complex. Treatment with SDS produced the characteristic full-length linear form of newly replicated plasmid DNA and even further cleavage resulting in a smear of linear products (Fig. 4A). Therefore, the cleavable complex is formed under conditions where replication is not inhibited. Up to 70% of plasmid DNA was converted into a linear form by 5 µg/ml antibiotic.

Gyrase as a norfloxacin target provided a control. The replication of plasmid DNA was shut down by only 0.02 µg/ml norfloxacin (Fig. 4B). This is most likely due to conversion of gyrase into a poison (15), because this concentration is too low to alter supercoiling of the plasmid (Fig. 4C). 50–100 times higher concentrations of the antibiotic are required to change the steady state level of plasmid supercoiling (33).

Thus, targeting of Topo IV with norfloxacin allowed replica-
tion of plasmid DNA, whereas targeting of gyrase immediately interfered with the progression of replication forks by formation of a drug-mediated gyrase-DNA complex. Because norfloxacin efficiently traps a Topo IV cleavage intermediate in vivo, however, we can also conclude that most Topo IV adducts turn over prior to collision with the replication fork. These results also suggest that the drug-stabilized Topo IV-DNA complex may be a less efficient barrier in front of the replication fork than the gyrase lesion. We conclude that the dissociation, repair, or bypass of the Topo IV-DNA complex contribute to the relatively slow killing via Topo IV (8). This is a major mechanistic difference from the gyrase complex, which efficiently and rapidly poisons replication.

Poisoning versus Catalytic Inhibition, as Judged by Genetic Dominance Relationships—When both wild-type and resistant alleles of parC are equally expressed in bacteria, cells acquire an intermediate level of drug resistance; the alleles co-domininate (8). This is an important difference from the unconditional dominance of the wild-type gyrA allele (1). The results with gyrase are readily explained within the classic poising framework; a single gyrase lesion can be lethal even in the presence of a vast excess of enzymatically active, drug-resistant gyrase. We conducted a comprehensive study of dominance relationships of parC alleles and directly compared the results with those with gyrA. The data indicate that Topo IV is also converted into a poison by the antibiotic.

The parC+, parC+, gyrA+, and gyrA+ genes were put under the control of the parC promoter. In addition to the chromosomal copy, these genes were on a pACYC184-based plasmid (copy number about 10) or a P1-based vector (copy number about 1). The MIC of strains with varying ratios of sensitive to resistant alleles is summarized in Fig. 5. We began by repeating past work on dominance of gyrase and Topo IV alleles. As expected, the wild-type gyrase allele (gyrA+ strain), when present on either the chromosome or a plasmid, determined the susceptibility of the bacteria to norfloxacin (Fig. 5, boxes A and B). The presence of even 10 copies of gyrA+ is irrelevant as long as a single gyrA+ is present. In contrast, the results with Topo IV ( gyrA+ strains) showed the expected co-dominance (box C).

Next, dominance experiments were conducted in repair (boxes D–K) mutant backgrounds. We first tested mutations in the recA gene because they had the most pronounced effect on the norfloxacin sensitivity of both gyrase and Topo IV (Fig. 3). A decrease in the repair of DNA damage caused by topoisomerase-mediated cleavage could elevate the number of lesions above the threshold level for killing. When RecA function was abolished either by deletion of the whole gene (DrecA, Fig. 5, box D) or by a point mutation (recA13, box E), a single wild-type allele of parC made the strain sensitive to norfloxacin even in the presence of a 10-fold excess of parC+ (compare half-filled and filled symbols in box D). Thus, recA mutations did render sensitivity dominant.

Mutations in recA are highly pleiotropic because of the multiple roles of the protein. To try to understand which aspect of RecA-controlled repair is responsible for the unconditional dominance phenotype, we tested several mutations of recA that selectively inactivate the repair activities of RecA in combination with mutations in other repair genes. None of the mutants tested was able to render sensitivity dominant as effectively as recA loss of function mutations. The mutants examined include the recA228 mutation (Fig. 5, box F), which has been reported to permit full scale SOS induction but renders the cells deficient in recombination (23). We also tested the lexA3 mutant (box G), which is defective in induction of the SOS repair pathway but is otherwise recombinationally proficient. Although both lexA3 and recA228 mutations made strains more susceptible to the drug in the presence of the parC wild-type allele (Fig. 5; compare sensitive/resistant S:R = 1:1, S:R = 1:10 in boxes F and G to the values in box C), their effect was reproducibly much less pronounced than DrecA. More importantly, the co-dominance of the resistant and sensitive alleles of parC was retained in these strains. The recB21 mutation, which eliminates SOS induction by quinolones (26, 27) (Fig. 2) and homologous recombination, also did not change the domi-
nance relationships (box H). The recN gene, which is specifically involved in the repair of double-strand breaks caused by x-rays (34), had no effect (data not shown). UvrA and uvrD mutants also did not make wild-type parC dominant (Fig. 5, boxes I and J). The status quo was preserved even in the recA428uvrD double mutant (Fig. 5, box K).

An important clue to understanding these results is that the ability to cause dominance of sensitivity among repair-deficient strains correlated with the degree of norfloxacin sensitivity on slow growth medium. Thus, only the RecA knockouts had a profound effect on the viability of Topo IV-targeted cells, and only these caused the dominance of wild-type Topo IV. Bacteria become more sensitive to chromosome loss in the absence of RecA (35), and such a loss can result from a spontaneous breakage of a chromosome followed by nuclease degradation. We suggest that in recA− cells the chromosome breakage induced by the Topo IV-drug-DNA ternary complex increases above the threshold that can be tolerated by the cell. This effect should be the most pronounced when the cell ploidy is minimal, i.e., on a slow growth medium, as was found.

Overexpression of the topoisomerase target should lead to increased antibiotic susceptibility (28) if the target subunit is limiting because more adduct results from a given drug concentration. In contrast, overproduction of the target of a catalytic inhibitor should decrease inhibition, because the increased enzyme concentration should overwhelm the effect of the drug. By comparing open symbols for gyrA+ and gyrA−ΔrecA strains in Fig. 5A (boxes A and B), it is clear that overexpression of GyrA makes strains much more sensitive to norfloxacin, as expected. Topo IV was again different. Overexpression of ParC does not lead to increased sensitivity in wild-type (see open symbols in box C) or in repair-deficient backgrounds (boxes D–K). Even in recA mutants, where a sensitive allele of parC dominates over a resistant one, overexpression of parC did not cause higher drug sensitivity.

Modulation of Dominance Relationships by Replication Mutants—The second set of mutants chosen in the search for external factors that might affect co-dominance of parC alleles were replication mutants. Replication mediates topoisomerase poisoning by converting the topoisomerase-DNA adduct into an irreversible lesion (19, 32, 36). Replication of plasmid DNA reconstructed in vitro in the presence of Topo IV is blocked by a norfloxacin-mediated Topo IV-DNA adduct (19). However, we demonstrated above that elongation of plasmid DNA is relatively insensitive to Topo IV targeting by a drug. This could be caused by a Topo IV-DNA cleavable complex being a poor barrier for the replication fork in vivo and/or by the dissociation before passage of a fork. If Topo IV works at the chromosome segregation stage, after replication is mostly completed, or supports replication from behind the replication fork (37), then the frequency of collisions between the Topo IV adduct and replication fork will depend on the half-life of the adduct. If the Topo IV-DNA complex dissociates faster than the minimal time required for the replication fork to reach the adduct, then collisions between them would be rare.

Therefore, if the time-space relationships between formation of the Topo IV catalytic complex and the replication fork were reversed, Topo IV should be converted into a poison that interferes with the progression of replication forks. This possibility was tested with two mutations. One is ΔseqA, which results in unscheduled replication (38). In this case, Topo IV behind one fork would be immediately ahead of another fork that fired prematurely from the origin of replication. The second mutant is gyrB236ts (39), whose temperature sensitivity was partially

**Fig. 5. Dominance of norfloxacin resistant ParC and GyrA in repair or replication mutant backgrounds.** Bacterial cells co-expressing GyrA (boxes A and B) or ParC (boxes C–M) wild-type (S) or resistant (R) subunits at the indicated ratios were plated on LB agar containing norfloxacin. Symbols within each box represent data averaged over at least three independent experiments. The relevant genotypes of each strain of the isogenic set are shown above the box. All strains are also gyrA+ unless specified otherwise. The MICs indicated by the dashed open circles and squares were lower than 0.001 μg/ml norfloxacin for both the gyrA+ and gyrA−ΔrecA strains. ○, wild-type allele expressed from pACYC based plasmid in the presence of resistant chromosomal copy; △, wild-type allele expressed from pACYC based plasmid in the presence of resistant chromosomal copy; ▼, only wild-type chromosome copy present; ▲, resistant allele expressed from P1-based plasmid in the presence of wild-type chromosomal copy; ■, resistant allele expressed from P1-based plasmid in the presence of resistant chromosomal copy; ●, only resistant chromosome copy present; □, resistant allele expressed from pACYC based plasmid in the presence of resistant chromosomal copy.

Expected ratio of wild type (sensitive) and resistant subunits

- 1S:10S
- 1R:10S
- 1S:0R
- 1S:1R
- 1R:1S
- 1S:10R
- 1R:0S
- 1R:10R
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The ratio of sensitive to drug-resistant ParC subunits of topo IV was varied as shown. The predictions of the poison model and the observed response to norfloxacin is indicated, with either ParE in excess over ParC or ParC in excess. m, concentration (Conc.) of ParE; n, concentration of ParC; S, wild-type level of sensitivity to norfloxacin; R, resistant level of sensitivity; S|R, sensitivity midway between S and R.

| Conc. of sensitive ParE (m) in excess (n > m) | Expected phenotype | Conc. of sensitive ParC (n) in excess (n > m) | Expected phenotype |
|---------------------------------------------|--------------------|---------------------------------------------|--------------------|
| 1.0                                         | S                  | m                                           | S                  |
| 0.1                                         | R                  | 0.04 μg/ml, ΔseqA gyrA' parC'              | R                  |
| 1.0                                         | S                  | 0.5 μg/ml, ΔrecA gyrA' parC'               | S                  |
| 1.1                                         | S                  | 2 μg/ml, ΔseqA gyrA' parC'                | S                  |

Effect of topo IV subunit ratios on the dominance of sensitive and drug-resistant forms

The efficient production of double-stranded breaks when Topo IV-targeted cells are treated with SDS contrasts with the inefficient killing of cells. This implies that the double-stranded breaks need to reach a threshold level for killing to occur. Topo IV has two subunits, ParC and ParE. If either subunit is in excess, the other is limiting for Topo IV activity. If a drug poisons an enzyme, the amount of sensitive enzyme determines the inhibition phenotype, because, in the face of a double-stranded break in DNA, the amount of still active resistant enzyme is irrelevant. (For catalytic inhibition, the situation is opposite, and the amount of resistant enzyme dictates phenotype.) Table II, we list the predicted consequences if either ParC or ParE is in excess for the five ratios of sensitive to resistant ParC that we have analyzed. The hypothesis is that ParE is in excess fails to explain any of the cases where the cell contains both sensitive and resistant alleles. The alternative that ParC is in excess explains well all of the results if we make the plausible assumption that when the amount of sensitive Topo IV falls by an order of magnitude, there is an insufficient amount of sensitive enzyme to reach the threshold for killing.

The last three rows of Table II in which both resistant and...
sensitive alleles are present are particularly illuminating. Because ParE is limiting, the presence of a 10-fold excess of resistant ParC in line 3 drives the amount of sensitive Topo IV to $\frac{1}{11}$ of the wild-type level, which we postulate is too low to result in cell killing. For gyrase, where conversion to the irreversible lethal lesion is much faster and more efficient, we conclude that this amount of sensitive enzyme is sufficient for killing. In line 4, the amount of sensitive allele is 10 times that of the resistant one. Therefore, the amount of sensitive enzyme is nearly the same as in wild-type cells, and the result is killing by norfloxacin. With an equal ratio of sensitive and resistant ParC, and therefore of sensitive and resistant enzymes (line 5), the killing efficiency of norfloxacin is midway between that in the wild-type and resistant cells.

This analysis also explains the hitherto puzzling dominance relationships seen with Staphylococcus aureus topoisomerase IV mutants (41). In S. aureus containing multiple copies of sensitive and quinolone-resistant ParC alleles, co-dominance was found, just as we had observed with E. coli. However, overexpression of both ParE and ParC resulted in unconditional dominance. Our interpretation is that the increase in ParE prevents the amount of sensitive Topo IV from dropping in the presence of a much larger amount of resistant ParC. The result is the dominance of the sensitive allele. We could not perform this experiment in E. coli, because overexpression of both subunits dramatically lowered viability (data not shown).

Our formulation readily explains the change in the dominance relationship we found in the repair and replication mutants of E. coli. The bellweather configuration is that of line 3 in Table II, in which there are 10 copies of the resistant allele to one of the sensitive. In wild-type cells, this results in drug resistance when Topo IV is the target, although killing is expected for classical poisoning. We tested eight repair mutants and found that only one recA knockout (recA13 and DrecA) brought about unconditional dominance of sensitivity. All of the other mutants did not significantly alter dominance. It is significant that the recA knockout caused the largest increase in killing by norfloxacin, 10–20-fold. We conclude that the great increase in killing efficiency is due to the lowered threshold level of Topo IV DNA adduct that leads to a lethal lesion. As a result, the drop of sensitive Topo IV to $\frac{1}{11}$ of its wild-type level was still sufficient for killing.

**Role of DNA Repair in Topo IV Targeting by Norfloxacin**—We conclude that a primary result of Topo IV targeting by norfloxacin is the formation of a topoisomerase-DNA cleavable complex, which can be converted into a double-stranded break. The cleavable complex is a bulky DNA adduct that should itself interfere with progression of the replication fork. Since the RecA and UvrABC repair pathways are the primary means of correction of these lesions, we analyzed their role in the repair of Topo IV-caused DNA damage.

Double-stranded breaks in DNA are repaired using the homologous chromosome as a guide. RecA is essential for this process. Indeed, we found that recA mutants are much more susceptible to Topo IV targeting by norfloxacin than their wild-type parents (Fig. 3). Moreover, in recA knockout strains, the wild-type parC allele dominated completely over the resistant one, which we interpret as resulting from greatly increased sensitivity to drug. Because we did not observe either suppression of the parC co-dominance or as large an increase in drug sensitivity in the recombination-deficient RecA mutant (recA428) or the repair-deficient recB21 and lexA3 mutants, we conclude that the phenotype of recA knockouts was not caused by the loss of either recombination or SOS induction alone. Instead, it required complete loss of RecA activities.

The unexpected repair phenotype of a recA mutant growing on aspartate minimal medium was illuminating. Such cells have only about 1 chromosome/cell/division (31), and damage correction via homologous recombination is limited by the small number of intact homologues. The SOS repair-deficient lexA3 mutants did not increase norfloxacin sensitivity on aspartate medium. The same was true for the recombination-deficient recA428 mutant. Nevertheless, DrecA caused an even more pronounced sensitivity (2–3-fold) to norfloxacin on aspartate medium versus LB medium. It seems likely that recA knockouts cannot tolerate the increased chromosome loss initiated by double-stranded breaks from Topo IV-DNA complexes. Such a loss occurs spontaneously in recA mutants.

**Poisoning of Cells via Quinolone Action on Topo IV**—The probability of collision between a replication fork and a Topo IV-DNA cleavable complex is equal to 1 if the half-life of the adduct is sufficiently long to be reached by the closest replication fork. The factors that affect the probability of the collision include the half-life of the adduct, the reparability of the adduct, the frequency of adduct formation, and the distance of the adduct from the replication fork.

We propose a model for inhibition of Topo IV that accounts for Topo IV as both a drug target and an enzyme (Fig. 7). The substrate for Topo IV is a catenane, precatenane, or a (+)-supercoiled node (42). When the topoisomerization reaction is interrupted by a quinolone and a Topo IV-DNA complex is formed, the complex can dissociate, be directly repaired, be converted into a double-strand break, and then be repaired or be hit by the replication fork. Because the majority of Topo IV molecules are still active at the low drug concentrations that are bactericidal (MIC is $\frac{1}{10}$ the $K_I$ value), all Topo IV substrates are still unlinked (Fig. 1). Relatively few Topo IV molecules are trapped on DNA in a potentially lethal cleavable complex. Any event that removes the adduct from the cycle shown in Fig. 7 leads to cell death. Collisions with the fork provide the major pathway to cell death. It has been shown in vitro that a Topo IV-DNA lesion becomes irreversible upon collision with the fork (19).

Collision between the fork and the adduct is a bimolecular reaction; its rate depends on the concentration of both the adduct and the fork. If the concentration of the adduct in front of the fork and the concentration of the fork behind the adduct are small, the probability of collision will be significantly increased when the concentration of either is increased. Our analysis above revealed that the ParC subunit is present in excess (Table II). Therefore, to increase the concentration of holoenzyme susceptible to drug, the ParE concentration has to be increased. This explains why overexpression of ParC alone does not increase drug sensitivity.

A similar analysis holds for the concentration of the replication fork. In two mutant strains, seqA and gyrB(topA20), the
probability of a fork immediately behind the Topo IV-DNA complex is increased. In the case of seqA cells, the increase is caused by increased replication initiation (38). We explain the gyrB results as follows. Because gyrase is impaired, (+)-supercoils now accumulate in front of the advancing replication fork. These are relaxed by Topo IV but now expose the cells to increased killing by quinolones, since Topo IV is now in front of the fork.

Support of this model is provided by our observation that Topo IV alone is sufficient to prevent accumulation of positively supercoiled plasmid DNA in the presence of quinolones. Thus, in both cases, albeit for different reasons, the frequency of collisions between the fork and the Topo IV-containing adduct will increase. If collisions occur frequently enough, one expects rapid inhibition of DNA synthesis after targeting Topo IV with drug. Indeed, seqA and gyrB mutations led to instant inhibition of DNA replication when Topo IV was inhibited. Thus, targeting of Topo IV can be gyrase-like with respect to the replication fork in some genetic backgrounds.

The original poisoning mechanism of inhibition by quinolones was described for DNA gyrase in the phage T7 system (15). There, gyrase was shown to be nonessential for replication of the phage DNA but nevertheless could be converted into an efficient poison of T7 multiplication by blocking its DNA replication in the presence of drug. Topoisomerase IV is also not required for DNA synthesis in E. coli but, nevertheless, can be converted by norfloxacin into a DNA-protein adduct capable of blocking replication. As a result, it kills the bacteria. This defines targeting of Topo IV by norfloxacin as, after all, a classical type of poisoning inhibition.

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