Topoisomerase IV, not gyrase, decatenates products of site-specific recombination in Escherichia coli

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DNA replication and recombination generate intertwined DNA intermediates that must be decatenated for chromosome segregation to occur. We showed recently that topoisomerase IV (topo IV) is the only important decatenase of DNA replication intermediates in bacteria. Earlier results, however, indicated that DNA gyrase has the primary role in unlinking the catenated products of site-specific recombination. To address this discordance, we constructed a set of isogenic strains that enabled us to inhibit selectively with the quinolone norfloxacin topo IV, gyrase, both enzymes, or neither enzyme in vivo. We obtained identical results for the decatenation of the products of two different site-specific recombination enzymes, phage λ integrase and transposon Tn3 resolvase. Norfloxacin blocked decatenation in wild-type strains, but had no effect in strains with drug-resistance mutations in both gyrase and topo IV. When topo IV alone was inhibited, decatenation was almost completely blocked. If gyrase alone were inhibited, most of the catenanes were unlinked. We showed that topo IV is the primary decatenase in vivo and that this function is dependent on the level of DNA supercoiling. We conclude that the role of gyrase in decatenation is to introduce negative supercoils into DNA, which makes better substrates for topo IV. We also discovered that topo IV has an unexpectedly strong DNA relaxation activity that, together with gyrase and topo I, is able to set the supercoiling levels in Escherichia coli.

[Key Words: DNA supercoiling; quinolones; λ integrase; Tn3 resolvase; topoisomerase I]

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The role of gyrase in decatenation is to maintain negative supercoiling to decatenate the negatively supercoiled catenanes. In a gyrase mutant, the partial activity of topo IV was sufficient to decatenate the recombination catenanes as they were no longer supercoiled because of DNA relaxation by topo I. The residual topo IV was insufficient to decatenate the products of recombination, we made the surprising discovery that topo IV has a strong DNA relaxation activity in vivo that, with gyrase and topo I, sets the cellular DNA supercoiling level. It was thought previously that only gyrase and topo I carried out this essential function (for review, see Wang, 1991).

**Results**

**Effect of topo IV and gyrase on Int recombination in vivo**

Our first series of experiments employed the Int recombination reaction. This site-specific recombinase activity is mediated by resolvase (Spengler et al. 1985). In vitro, these catenanes are quickly unlinked by type-2 topoisomerases into free circles (Bliska and Cozzarelli 1987). The reaction protocol we used to determine which enzyme unlinks the Int products is schematized in Figure 1. The strains were lysogens for a mutant phage λ, which contains the Int gene under control of the temperature-sensitive repressor, cl857 (Bliska and Cozzarelli 1987). The strains harbored the Int recombination substrate pB3.5d (Bliska and Cozzarelli 1987). Int expression was initiated by shifting the cultures to 43°C for 10 min to inactivate the repressor. Norfloxacin was added (or not) and the cultures were shifted back to 30°C to activate the thermolabile wild-type Int protein (Guarnieros and Echols 1973). Thus, recombination of the plasmid substrate and topoisomerase inhibition were initiated at the same time (time 0). Cultures were incubated at 30°C for up to 60 min. If the topoisomerase responsible for decatenation is drug inhibited, then the recombinant plasmid DNA would be trapped as catenanes.

Before we consider the fraction of products that is catenated, it is important to note that norfloxacin affects the frequency of recombination. The inhibition of topoisomerases not only blocks decatenation, but also influences the level of cellular DNA supercoiling, which, in turn, modulates recombination (Nash 1990). Because su-
percoiling affects transcription [indeed, even of the topoisomerases themselves (Menzel and Gellert 1983)], we limited transcription of Int to the time before the addition of norfloxacin to ensure that the resultant changes in DNA supercoiling have no effect on Int synthesis, only on the ability of Int to recombine (Fig. 1).

We measured the effect of norfloxacin on the extent of recombination in each of six test strains, LZ33–LZ38 (see Table 1). For convenience, we refer to these strains by the topo IV and gyrase genotypes. Plasmids were isolated at various times throughout the recombination reactions, nicked with DNase I, and analyzed by high resolution gel electrophoresis and Southern blotting. The amount of label in the recombinant products as a percentage of the total label in all plasmid DNA for each strain is shown in Figure 2. In this and two subsequent figures, the results are presented as bar graphs. The three panels on the left are gyrA+ strains and the three on the right are strains with gyrA183, which renders gyrase highly norfloxacin resistant. In the top row, parC is wild type; in the middle row, parC is resistant to norfloxacin to an intermediate degree (parC180); and in the bottom row, parC is highly resistant to norfloxacin (parC184). For each strain, we show the amount of recombination in the presence of 0, 30, 60, 90, or 120 µM norfloxacin. For each drug concentration, data are shown for 0, 20, 30, 45, and 60 min after drug addition and shift down to 30°C. The drug concentrations were chosen based upon the Kᵢ values that we determined for the inhibition of topo IV or gyrase in vitro and in vivo (Table 2A; see Materials and Methods). From these Kᵢ values, we constructed Table 2B showing qualitatively the inhibition of wild-type or mutant gyrase and topo IV at the concentrations of drug used in this work.

In the absence of norfloxacin, the extent and rate of recombination were the same for each strain (Fig. 2, (Nor) = 0), showing that the topoisomerases mutants have the same Int expression and activity as the wild-type strain. When gyrase was drug-resistant and thus able to maintain negative supercoiling, recombination occurred at all drug concentrations tested (Fig. 2, right panels). The drug resistance imparted by this gyrA allele exceeded 180 µM norfloxacin (Table 2; data not shown).

In contrast, when gyrase was wild type, and thus inhibited by norfloxacin, recombination was clearly reduced by even the lowest drug concentration used, 30 µM (Fig. 2, cf. left and right panels in each row). The surprising result was that the inhibition of recombination by norfloxacin was greatly affected by the drug resistance of topo IV (Fig. 2, left panels). When parC was wild type,

### Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Construction or Reference</th>
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<tbody>
<tr>
<td>CAG12183</td>
<td>zei-3143::Tn10kan</td>
<td>Singer et al. (1989)</td>
</tr>
<tr>
<td>C600</td>
<td>F- thr-1 leu-6 thi-1 lacY1 supE44 tonA21</td>
<td>Kato et al. (1988)</td>
</tr>
<tr>
<td>KL16</td>
<td>Hfr thi</td>
<td>Yoshida et al. (1988)</td>
</tr>
<tr>
<td>LZ1</td>
<td>Hfr thi gyrA183 zei-723::Tn10</td>
<td>Zechiedrich and Cozzarelli (1995)</td>
</tr>
<tr>
<td>LZ2</td>
<td>Hfr thi zei-723::Tn10</td>
<td>Zechiedrich and Cozzarelli (1995)</td>
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<td>Khodursky et al. (1995)</td>
</tr>
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<td>C600 except zei-723::Tn10 parC180, kanR</td>
<td>Khodursky et al. (1995)</td>
</tr>
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<td>C600 except gyrA183 zei-723::Tn10 kanR</td>
<td>Khodursky et al. (1995)</td>
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<tr>
<td>LZ24</td>
<td>C600 except zei-723::Tn10 kanR</td>
<td>Khodursky et al. (1995)</td>
</tr>
<tr>
<td>LZ27</td>
<td>LZ21 except Tet5</td>
<td>fusaric acid6</td>
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<td>LZ22 except Tet5</td>
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<td>LZ29</td>
<td>LZ23 except Tet5</td>
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<td>P1(LZ1) x W3101ΔΔ, TetR</td>
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</tr>
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<td>LZ32 except parC180, kanR</td>
<td>P1(LZ22) x LZ32, KanR</td>
</tr>
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<td>LZ35</td>
<td>LZ31 except kanR</td>
<td>P1(LZ24) x LZ31, KanR</td>
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<td>P1(1644) x LZ31, KanR</td>
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<td>LZ54</td>
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<td>P1(LZ50) x RS2Δ, KanR</td>
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<tr>
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<td>Hfr thi gyrA183</td>
<td>Yoshida et al. (1988)</td>
</tr>
<tr>
<td>RS2Δ</td>
<td>pyrf287 mirA trpR7 cldR7 gal25 rpsL195 top10 λ (P80 xis1 cI857)</td>
<td>Bliska and Cozzarelli (1987)</td>
</tr>
<tr>
<td>W3101ΔΔ</td>
<td>F-λ (P80 red114 xis1 cI857)</td>
<td>Bliska and Cozzarelli (1987)</td>
</tr>
<tr>
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<td>Khodursky et al. (1995)</td>
</tr>
<tr>
<td>1644</td>
<td>C600 except zei-723::Tn10 parC184, kanR</td>
<td>Khodursky et al. (1995)</td>
</tr>
</tbody>
</table>

*Constructions are shown as chemical used for selection or as donor x recipient, selection. Abbreviations: (R) Resistant; (S) sensitive; (Tet) tetracycline; (Kan) kanamycin; (P1) source of transducing phage lysate.
recombination was decreased, but still robust in the gyrA\(^+\) strain, compared with the isogenic gyrA\textit{L83} strain. Recombination was reduced with drug in the parC\textit{L80} strain and even further reduced in the presence of the more resistant parC\textit{K84} allele.

The most direct explanation for these results is that topo IV lowers the (−) supercoiling density of the substrate plasmids. The Int reaction studied here requires (−) supercoiled DNA (Nash 1990) and uninhibited topo IV relaxes the plasmid DNA and makes it an inferior substrate for Int. Accordingly, DNA relaxation by topo IV is almost completely blocked by norfloxacin in wild-type parC strains, partially blocked in the strain carrying the intermediate drug-resistant parC\textit{L80} allele, and is barely affected in the parC\textit{K84} strain when gyrase is wild type. The dependence of recombination on drug concentration is best explained by the inhibition of topo IV DNA relaxation activity at the highest norfloxacin doses. Thus, without the topoisomerases to alter it, the DNA remains negatively supercoiled and highly recombinogenic.

Previously, the equilibrium balance of (−) supercoiling has been thought to be controlled in a simple fashion by the opposing action of gyrase and topo I (Gellert 1981; Pruss and Drlica 1982; Wang 1985; Liu and Wang 1987). DNA gyrase pumps (−) supercoils into DNA, whereas topo I selectively removes (−) supercoils. These functions are influenced by tracking processes such as transcription, which separates supercoiled DNA into (+) and (−) domains, and also depend upon DNA-anchoring sites that maintain these domains (Liu and Wang 1987; Cook et al. 1992; Lynch and Wang 1993). Previously, no role for topo IV in DNA supercoiling was observed (Wang 1991; Adams et al. 1992b; Khodursky et al. 1995; Zechiedrich and Cozzarelli 1995). However, topo IV is known to relax DNA in vitro (Kato et al. 1992) and in vivo when overexpressed (Free and Dorman 1994; McNairn et al. 1995).

Effect of topo IV and gyrase on DNA supercoiling levels in vivo

To test directly whether normal levels of topo IV have an effect on DNA supercoiling in vivo, we examined a portion of the DNA samples described in Figure 2 (before they were nicked) on agarose gels containing various concentrations of chloroquine. Chloroquine untwists circular DNA in a controlled manner and thereby allows the electrophoretic separation of DNA topoisomers and the measurement of supercoiling density (\(\kappa\)) (Keller 1975).

**Decatenation by topo IV in vivo**

![Figure 2. Effect of norfloxacin on Int recombination. The experiment was as described in the legend of Fig. 1. Plasmid DNA was isolated, subjected to electrophoresis on high resolution agarose gels, Southern blotted, and quantified by PhosphorImager analysis. The total amount of plasmid DNA label was normalized to 100%. The percentage of the total recombined plasmid is shown for various time points and drug concentrations (Nor) in each of the six strains. Time after shift down to 30°C is represented as black wedges. Samples were analyzed at the following times after the addition of drug: 0 min (■), 20 min (squares in box), 30 min (white box), 45 min (hatched box), and 60 min (dotted). The graphs are presented in a matrix array depending upon the states of the topo IV and gyrase alleles. (parC\textit{+} gyrA\textit{+}) LZ36; (parC\textit{+} gyrA\textit{L83}) LZ35; (parC\textit{L80} gyrA\textit{+}) LZ34; (parC\textit{L80} gyrA\textit{L83}) LZ33; (parC\textit{K84} gyrA\textit{+}) LZ38; (parC\textit{K84} gyrA\textit{L83}) LZ37. (N.D.) Not determined. Parts of these experiments were repeated three times. The exact values varied somewhat, but the relative values were always the same. For example, the 30 µM drug, 20 min point for the double wild-type strain was done three times. The amount of recombination averaged 30% ±7% and the amount of catenation averaged 75% and varied ±4%. The results here and in Figs. 3 and 5 are from one extensive experiment performed all on the same day.](https://genesdev.cshlp.org/figure2.png)
Gels were blotted and the results were analyzed with a PhosphorImager. The results are presented as a histogram similar to Figure 2 except the ordinate shows $s$ (Fig. 3). Completely relaxed DNA has a $s$ value of 0. In the absence of drug, the $s$ for plasmid pJB3.5d in all strains was $-0.075$ (left-most set in each panel). This shows that the topoisomerases in the mutant strains are functioning equally in the absence of drug. When $gyrA$ was drug-resistant, norfloxacin had little effect on $s$ (Fig. 3, right panels). When $gyrA$ was wild type, however, the supercoiling level was reduced to a degree dependent upon the $parC$ allele (Fig. 3, left panels). The more drug-resistant the $parC$ allele was, the greater the observed DNA relaxation. Therefore, the decrease in recombination seen in Figure 2 is caused by the simultaneous inhibition of (-) supercoiling by DNA gyrase and the rapid DNA relaxation by drug-resistant topo IV (and topo I). Further, in the wild-type strain, topo IV is inhibited by norfloxacin and only topo I relaxes the plasmid DNA (Fig. 2, top left). Topo I alone, then, must not be able to relax DNA beyond $s \sim -0.05$ in vivo. Topo IV, on the other hand, can relax the DNA to near completion ($s \sim -0.012$) in the drug-resistant $parC$ strains.

To illustrate the effect of (-) supercoiling on Int recombination, we plotted the percentage of recombination as a function of $s$ for all the data in Figures 2 and 3 (Fig. 4). The transition from a baseline level of recombination to maximal recombination is surprisingly sharp and the midpoint lies around a $s$ of $-0.05$. This supercoiling level required for recombination is near measured wild-type $s$ levels for various plasmids and the chromosome (Sinden et al. 1980; for review, see Wang 1984). Clearly, Int requires highly (-) supercoiled DNA in vivo and this DNA is modulated by topo IV relaxation.

Topo IV decatenates products of Int recombination in vivo

Even when $s$ was lowest (when gyrase was inhibited and topo IV was not), recombination could be detected. Thus, decatenation of the recombination products could be measured. The high-resolution gels used for Figure 2 allow separation of the catenanes from both unreacted Int substrate plasmids and unlinked products. From these data, we measured the amount of recombination (catenanes plus free circles) for each time point and normalized it to 100%. Of that, the amount of recombined plasmid DNA that was catenated was plotted for each strain (Fig. 5). The histogram panels are as in Figures 2 and 3. In the absence of drug, no appreciable amount of catenanes was seen at any time point. Nearly all of the plasmids that were recombined in the $parC^+$$gyrA^+$ strain remained as catenanes when norfloxacin was present, even 60 min after recombination was initiated (Fig. 5, upper left). At the other extreme, the double drug-resistant mutants, $parC^{L80}$$gyrA^{L83}$ or $parC^{K84}$$gyrA^{L83}$, showed very few catenanes with drug, about 10% (middle and lower right panels). These data establish that topo IV and gyrase are the only relevant decatenases of the products of Int recombination in E. coli.

Table 2. Effects of norfloxacin on topo IV and DNA gyrase

<table>
<thead>
<tr>
<th>Strain</th>
<th>30 µM</th>
<th>60 µM</th>
<th>90 µM</th>
<th>120 µM</th>
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<td>gyrA</td>
<td>parC</td>
<td>gyrA</td>
<td>parC</td>
<td>gyrA</td>
</tr>
<tr>
<td>wt</td>
<td>wt</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>wt</td>
<td>L80</td>
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<td>+</td>
<td>−</td>
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<tr>
<td>wt</td>
<td>K84</td>
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<td>L83</td>
<td>wt</td>
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<tr>
<td>L83</td>
<td>K80</td>
<td>+</td>
<td>+</td>
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</table>

Note: The concentration of norfloxacin is shown; −, +/-, and + correspond to complete, partial, and no inhibition, respectively, of the enzyme in the strain composed of the gyrA and parC alleles indicated in the first two columns. (wt) Wild type.

### A. $K_i$ values for gyrase and topo IV

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>In vitro $K_i$ (µM)</th>
<th>In vivo $K_i$ (µM)</th>
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<tbody>
<tr>
<td>gyrase</td>
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<td>7.6</td>
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<td>topo IV</td>
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### B. Inhibition of gyrase and topo IV

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<th>gyrA</th>
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<th>gyrA</th>
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Note: Measure by DNA supercoiling

Note: Measured by decatenation

Table 2. Effects of norfloxacin on topo IV and DNA gyrase

A. $K_i$ values for gyrase and topo IV

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>In vitro $K_i$ (µM)</th>
<th>In vivo $K_i$ (µM)</th>
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<td>Wild type</td>
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<td>GyrA$^{L83}$</td>
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B. Inhibition of gyrase and topo IV

<table>
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</table>
parC, gyrA + (bottom left) have substantially lower levels of catenanes than the strain with drug-resistant gyrase (parC + gyrA L83). At higher drug concentrations, first the parC L80 and then the parC K84 drug-resistance is partially overcome, as indicated by the increase in catenanes at 90 µM and 120 µM norfloxacin, respectively. These results are expected if topo IV, not gyrase, is the primary decatenase of recombination products.

Gyrase plays some role in decatenation of recombination products because there is roughly a 30% difference in the levels of catenation between the parC + gyrA + and parC + gyrA L83 strains (Fig. 5, cf. top left and right panels). Gyrase may directly decatenate this fraction of catenanes. Alternatively, because topo IV unlinks supercoiled catenanes four-fold better than it unlinks relaxed catenanes in vitro (Ullsperger and Cozzarelli 1996), it is possible that the role played by gyrase is to boost the decatenation activity of residual uninhibited topo IV by supercoiling the plasmids. As a result, when supercoiling is normal (in gyrA L83 strains), residual topo IV decatenates more efficiently than it does when the DNA is relaxed.

To visualize better the relationship between supercoiling (s) and decatenation, we plotted this dependence for every data point from Figure 5 for parC + strains, in which catenanes are preserved by drug addition. As with recombination (Fig. 4), s affects strikingly the amount of decatenation (Fig. 6). There is an abrupt decrease in the fraction of catenanes over the relatively small supercoiling density range (~0.05 to ~0.075).
We carried out the following experiment to test whether the role of DNA gyrase is to introduce negative supercoils into the DNA, which is then decatenated by topo IV exclusively. We reasoned that if this hypothesis is correct, then if we could block DNA relaxation, the inhibition of gyrase would not result in a loss of supercoiling. The consequence would be that recombination, supercoiling, and catenation should now be the same in the gyrA\(^+\) and gyrA\(^{L83}\) strains after the treatment with norfloxacin. Although we knew from the data in Figures 2 and 3 that topo IV was able to relax DNA in the presence of topo I, we expected the role of topo I to predominate because it has been considered to be the primary (−) supercoil relaxation enzyme in the cell (for review, see Wang, 1991). We moved our drug-resistant and wild-type parC and gyrA alleles into a strain RS2\(_l\), which contains a mutation in topo I, top10, which reduces topo I activity 100-fold (DiNardo et al., 1982).

The experiment proved to be more complex than we had anticipated, but for an interesting reason. After inhibition of gyrase by drug, topo IV alone was able to relax the DNA to near completion in the topo I mutant (data not shown). Although this result confounded our original intent, we obtained useful information. In these experiments, topo IV was wild-type and topo I was mutant. In the presence of drug, there was a fourfold faster initial rate of recombination in the gyrA\(^{L83}\) strain than in the gyrA\(^+\) control (Fig. 7A). In addition, recombination reached 95% in the strain with drug-resistant gyrase, but never exceeded 50% in the wild-type strain. Even without topo I activity to relax the DNA, the level of DNA supercoiling was significantly reduced compared to the wild-type control. This result suggests that topo IV is capable of relaxing DNA even in the absence of topo I, and that the inhibition of gyrase is not solely due to a loss of supercoiling.

Figure 5. Effect of norfloxacin on decatenation of Int recombination products. The amount of recombined DNA from Fig. 2 was further broken down into catenanes and free circles (decatenated). The percentage of the total recombined plasmid that was catenated is shown.

Figure 6. Effect of DNA supercoiling (σ) on decatenation. The correlation between decatenation and σ is shown for every parC\(^+\) data point in Fig. 5.
supercoiling was dependent upon gyrase activity. The value of $\sigma$ after the addition of drug was $-0.053$ in the strain with wild-type gyrase, but reached the very high level of $-0.093$ in the gyrA$^{L83}$ strain. Therefore, through its effect on $\sigma$, gyrase has an effect on recombination.

Our chief interest is in the portion of recombined DNA that was catenated (Fig. 7B). The majority (70%–90%) of the DNA remained catenated after drug treatment, but as in Figure 5, there was $\sim20\%$ difference in catenation depending on whether gyrase was drug resistant. This difference is small compared with the differences in recombination and $\sigma$, however. With time, the catenanes are unlinked slightly in both strains. As revealed from the slopes of the least squares fit lines of the kinetic data, the unlinking activity when gyrase is drug resistant is $\sim3.5$-fold greater than the unlinking activity when gyrase is wild type. For comparison, we show the rate of catenane turnover when topo IV is active and gyrase is not in a parC$^{K84}$ gyrA$^+$ strain. This rate is $\sim25$-fold faster than when gyrase alone is active. Therefore, the gyrase contribution to the rate of decatenation is far less than that of topo IV. Overall, these results are the same as those for the wild-type topo I strains and indicate that there are two rates of decatenation—an efficient unlinking by uninhibited topo IV and a rate $\sim25$-fold slower, which we believe is carried out by incompletely inhibited topo IV. Residual topo IV is boosted by the high level of negative supercoiling in the gyrA$^{L83}$ strain.

Decatenation by topo IV in vivo

The most common plasmid catenate type in E. coli is the singly linked catenate (Wasserman and Cozzarelli 1986). A singly linked (two-noded) catenate can arise from DNA replication, type-2 topoisomerization, or recombination (Wasserman et al. 1988). The two-noded catenate is structurally unique among catenanes in that there is no strain in the DNA introduced by the link (Wasserman and Cozzarelli 1986). Therefore, there is not the same enthalpic drive to decatenation as with multiply interlinked catenanes. Because of this and because of the complexity of the results with Int, an independent evaluation of the roles of topoisomerases in unlinking recombination catenanes was desirable. Therefore, we tested whether topo IV also played the key role in the unlinking of the two-noded catenanes formed by Tn3 resolvase.

As with Int, resolvase expression was under the $\lambda$ phage $\rho$ promoter controlled by the temperature-sensitive cI857 repressor (Bliska et al. 1991). There were two differences between the Int and resolvase systems (Fig. 8). First, because resolvase is active at $30^\circ$C and then shifted to $43^\circ$C to induce resolvase expression, the enzyme recombines the two directly repeated sites on the substrate plasmid DNA and the product is a singly linked (two-noded) catenate. The sizes of the rings were 2.75 and 3.2 kb. A type-2 topoisomerase unlinks the catenanes to generate free circles. Drug (norfloxacin) was added after recombination but before most unlinking.

**Figure 7.** Kinetics of recombination and decatenation. (A) Rate of recombination in top10 parC$^+$ gyrA$^+$ (LZ53; ●) and top10 parC$^+$ gyrA$^{L83}$ (LZ54; ○) strains. The experiment was as outlined in Figs. 1 and 2. The drug concentration was 90 µM. (B) Amount of catenation in top10 parC$^+$ gyrA$^+$ (●) and top10 parC$^+$ gyrA$^{L83}$ (○) strains. Catenane turnover in a parC$^{K84}$ gyrA$^{L83}$ strain (LZ37; ■) is shown for comparison. From the relative slopes, catenane unlinking when gyrase is active and topo IV is inhibited (○) is 3.5-fold faster than when both enzymes are inhibited (●) and 25-fold faster than when topo IV is uninhibited (■).
were both resistant to drug (mid was catenated. In contrast, when topo IV and gyrase concentration. Two strains were assayed: topo IV, unlinks supercoiled in up to 200 µM norfloxacn (data not shown). In the absence of drug, the rate of resolvase recombination and subsequent decatenation was identical in all strains (data not shown). With increasing concentrations of norfloxacn, increasing amounts of recombination products were trapped as catenanes when topo IV alone was inhibited (parC+ gyrA L83) (Fig. 9). At 30 µM, there was only a slight block of decatenation. At 60 µM, half of the recombined plasmid was catenated. In contrast, when topo IV and gyrase were both resistant to drug (parC+L80 gyrA L83), there was a rapid unlinking of the catenanes into free circles even with norfloxacn as high as 300 µM. Although we could make no reliable quantitation, we observed catenanes in the parC+ gyrA L83 strain and not in the parC+L80 gyrA L83 strain (data not shown). We conclude that topo IV is also the primary decatenase of singly linked recombination products.

**Discussion**

By use of isogenic strains with various combinations of alleles encoding wild-type and drug-resistant gyrase and topo IV, we have determined the division of labor of the type-2 topoisomerases in the cell. We find that topo IV, not gyrase, is the enzyme that unlinks the catenated intermediates of two different recombinases. Our conclusion that topo IV is the primary decatenase of recombination intermediates differs from the earlier conclusion that DNA gyrase was responsible (Bliska and Cozzarelli 1987; Bliska et al. 1991). Our data agree with the earlier results, but because they are far more extensive, lead to the revised interpretation. These results, combined with our previous work with DNA replication intermediates (Zechiedrich and Cozzarelli 1995), allow us to conclude that topo IV is the primary enzyme for all decatenation in the cell.

The role of gyrase in decatenation

Although topo IV is the major unlinking enzyme, gyrase does have an effect on decatenation. This is apparent from the reduction in decatenation after inhibition of gyrase with drug (Figs. 5 and 7). There are two ways that gyrase could promote decatenation. First, gyrase might directly unlink catenanes, but at a rate 25-fold lower than that of topo IV (see Fig. 7). This contribution is similar to what we previously estimated for DNA gyrase decatenation of replication intermediates, which was roughly 100-fold less than the contribution of topo IV (Zechiedrich and Cozzarelli 1995). The weak activity of gyrase was not sufficient to prevent catenation of replicated plasmids and a partition defect phenotype of conditionally lethal topo IV mutants (Kato et al. 1988; Schmid 1990).

The second possibility is that gyrase negatively supercoils the catenanes, which makes them superior substrates for topo IV decatenation. There are three arguments in favor of this possibility. (1) Purified topo IV unlinks supercoiled catenanes at least fourfold faster than relaxed catenanes in vitro (Ullsperger and Cozzarelli 1996) and at equilibrium supercoiling favors plasmid decatenation by more than two orders of magnitude (Rybenkov et al. 1997). The reduction in decatenation after norfloxacn addition to the gyrA L83 strain compared with the resistant mutant in all experiments correlated with σ value; (−) supercoiling promotes decatenation (Fig. 6). (2) If drug-resistant gyrase were directly decatenating some fraction of the catenanes, then the inhibition of topo IV should not alter that fraction. Instead, decatenation is far better when topo IV is drug resistant (Fig. 5). If anything, the presence of a drug-resistant topo IV should impair any direct decatenation by gyrase because topo IV relaxes the plasmid DNA and gyrase was also found to unlink supercoiled substrates better than relaxed substrates in vitro (Ullsperger and Cozzarelli 1996). (3) The turnover of the catenanes is blocked in the parC+ gyrA L83 strain at the highest drug concentrations used (Fig. 5) even though gyrase is still fully functional [as evidenced by the complete recombination (Fig. 2) and unaltered supercoiling (Fig. 3) at the same drug concentrations]. Uninhibited gyrase should be able to continue to decatenate if it is responsible directly for decatenation.

If supercoiling by gyrase is inhibited, the activity of the drug-resistant topo IV on the relaxed DNA is still.
Decatenation by topo IV in vivo

The role of topo IV in DNA supercoiling

It has been long thought that only two topoisomerases, topo I and DNA gyrase, counteracted each other to maintain the level of DNA supercoiling in bacteria (for review, see Wang 1991). Topo I removes (−) supercoils and DNA gyrase introduces (−) supercoils. Here, we show that topo IV has a potent relaxing activity in vivo that almost completely removes (−) supercoils after the inhibition of gyrase (Fig. 3). The extent of DNA relaxation by topo IV (r = −0.012; Fig. 3, bottom left) exceeds the extent of relaxation by topo I (r = −0.055; Fig. 3, top left). The relaxation activity of topo IV was missing before because the drugs used to inhibit gyrase also inhibited topo IV, the same reason that the role of topo IV in decatenation of recombination intermediates was missed. Only when we used a drug-resistant topo IV were the new activities revealed. These results call for a re-examination of previous research on DNA supercoiling in bacteria and we have addressed the relative importance of the topoisomerases in DNA supercoiling in a separate study (E.L. Zechiedrich, A.B. Khodursky, and N.R. Cozzarelli, in prep.).

Our findings reveal a complete division of labor between topo IV and DNA gyrase. Through the same DNA strand-passage mechanism, these homologous topoisomerases perform radically different functions in the cell. Topo IV decatenates replication and recombination intermediates as well as relaxes DNA supercoils. DNA gyrase introduces (−) supercoils in DNA. We speculate that, when examined more closely, perhaps other enzymes that are considered to be redundant may also have distinct roles in vivo.

Materials and methods

Chemicals and reagents

\( \alpha^{32P} \) dCTP (250 mCi/m mole; 1 Ci/ml) was from NEN. The supercoiled DNA and 1 kilobase ladders were from GIBCO BRL. Proteinase K was from Boehringer-Mannheim. Norfloxacin, fusaric acid, chlorotetracycline, chloroquine, RNaseA, and DNAse I were from Sigma. Multiprime DNA-labeling kit was from Amersham and Nytran (MS). transfer membrane from Fisher.

Bacterial strains and plasmids

The bacterial strains used in this study are listed in Table 1. Four isogenic sets of strains containing all possible combina-

Inhibition of topo IV and gyrase with norfloxacin

To determine the drug sensitivity of the catalytic activity of the enzymes in vivo, we measured the amount of drug required to inhibit by 50% the supercoiling activity of gyrase and the decatenation activity of topo IV. Of course, quinolone antibiotics are not merely inhibitors of the topoisomerases. They act by the so-called poisoning mechanism whereby the cleaved DNA intermediate in the topoisomerase reaction is stabilized (Kreuzer and Cozzarelli 1979; Tevey et al. 1984). We do not include such covalent enzyme-DNA complexes in our analyses. However, they represent <5% of the plasmids (data not shown). Therefore, the change in plasmid topology levels that we analyze result
from the removal of the topoisomerase being tested. Steady-state supercoiling of plasmid pBR322 DNA was chosen to be the measure of gyrase activity. The $K_i$ value for gyrase was defined as the minimal concentration of antibiotic that caused a twofold decrease in the amount of the topoisomerase closest to the mean value.

A accumulation of DNA replication catenanes was used to determine the extent of topo IV inhibition by norfloxacain. The $K_i$ value for topo IV was defined as the minimal drug concentration at which half of the newly replicated DNA was catenated after a 0.5-min pulse (see Khodursky et al. 1995; Zechiedrich and Cozzarelli 1995). Measurement of the inhibition of topo IV was possible only in the presence of the drug-resistant gyrA because with wild-type gyrase, DNA replication was inhibited at drug concentrations much lower than those that inhibited topo IV (Khodursky et al. 1995). The $K_i$ values in vitro and in vivo are summarized in Table 2A. We were unable to determine the amount of drug needed to overcome the resistance of parC$^{L80}$ in vivo because the resistance of the gyrA$^{R83}$ allele was overcome first. For the same reason, we could only estimate the $K_i$ value for parC$^{L80}$. We used the $K_i$ values in vitro and in vivo (Table 2A) to assess the range of drug concentrations that would be active against the mutant enzymes in our experiments (Table 2B). For 30 $\mu$M or higher norfloxacain, wild-type gyrase was inhibited. The (+) supercoiling activity of drug-resistant gyrase in vivo was not inhibited until $>180$ $\mu$M norfloxacain. ParC$^{L80}$ was mostly inhibited at 30 $\mu$M and completely blocked at higher concentrations. ParC$^{L80}$ was unaffected at 60 $\mu$M and was inhibited at 90 $\mu$M or higher. ParC$^{K84}$ was slightly inhibited at 120 $\mu$M, but was not at the lower drug concentrations.

**Int recombination**

The assay used for Int recombination (Bliska and Cozzarelli 1987; Adams et al. 1992a) is schematized in Figure 1. Lysogens harboring the plasmid pB3.5d were grown at 30°C in LB with 50 $\mu$g/ml of ampicillin. At a cell density of 70 Klett units, cultures were shifted to 43°C to inactivate the temperature-sensitive $c$ phenotype. Norfloxacin was added to inhibit the active against the mutant enzymes in our experiments (Table 2B). Plasmids were isolated and analyzed as described above.

**Resolvase recombination**

The assay used for resolvase recombination was as described (Bliska et al. 1991) and is depicted in Figure 8. Cells harboring pBREsc1 and pRR51 were grown at 30°C to a density of 70 Klett units in LB with 30 $\mu$g/ml of chloramphenicol, 50 $\mu$g/ml of ampicillin, and 15 $\mu$g/ml of tetracycline. The cells were shifted to 43°C (time =0) to induce resolvase expression. Cultures were shaken for up to 10 min at 43°C. To block decatenation of resolvase recombination products, norfloxacin was added 140 sec after the upshift. This time for drug addition gave a maximal ratio of catenanes to free circles (data not shown). All plasmids (1.9 ml) were removed before upshift and at various times after shift to 43°C. Plasmids were isolated and analyzed as described above.

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