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Effects of Ciprofloxacin on Plasmid DNA Supercoiling of *Escherichia coli* Topoisomerase I and Gyrase Mutants

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Changes in plasmid DNA supercoiling were measured following treatment of *Escherichia coli* cells, carrying topoisomerase mutations, with the quinolone ciprofloxacin. In quinolone-susceptible cells (*top*$^+$ *gyr*$^+$) as well as in *topA* mutants and in *gyrB* mutants, plasmid DNA was relaxed after the addition of ciprofloxacin. In cells partially resistant to quinolones, low ciprofloxacin levels led to an increase in negative superhelicity of plasmid DNA, whereas at higher ciprofloxacin concentrations, DNA became relaxed. Cells exhibiting partial resistance to quinolones carried either a *gyrA* mutation alone or a combination of *gyrA* and *gyrB* mutations. Moreover, they showed a reduction in gyrase activity, indicated by the supercoiling of a reporter plasmid. Therefore, we conclude that a low level of quinolone action and a DNA with a lower-than-normal level of superhelicity are the two essential conditions for obtaining a ciprofloxacin-promoted increase in plasmid DNA supercoiling. In contrast, deficiency in topoisomerase I is not required for this effect.

Quinolone antibiotics are bactericidal agents that specifically inhibit DNA gyrase. This class of compounds includes the old quinolones, such as nalidixic and oxolinic acids, and the recently developed, more potent quinolones, such as ciprofloxacin and norfloxacin (for reviews, see references 10, 18, 20). The target of quinolones seems to be the complex formed by gyrase and the DNA. This complex is blocked by the binding of drug molecules to the single-stranded DNA pocket formed by the action of gyrase (19). Therefore, quinolones have been considered gyrase poisons and not simple inhibitors of gyrase activity (9, 11, 13).

The effects of quinolones upon the topological state of DNA appear to be rather complex. Oxolinic acid causes partial relaxation of the bacterial chromosome in both *top*$^+$ and *topA* cells (17), whereas it has been reported that plasmid DNA fails to be relaxed in *topA* mutants after treatment with norfloxacin (4). In the triple mutant GP200 (Δ*topA gyrA307 gyrB225*), which is resistant to only low levels of quinolones, oxolinic acid causes an increase in the supercoiling of chromosomal as well as plasmid DNA (12, 17).

This last result is intriguing because of the apparent requirement for three mutations affecting topoisomerases. However, the analysis of the role played by each mutation in the observed effect has proved to be difficult because of the lack of knowledge about the effects of the *gyrA307* allele on gyrase activity and the fact that Δ*topA* cells are viable only in the presence of *gyr* compensatory mutations (7) or when grown in a low-osmolarity medium (8).

We have recently reported the isolation of *gyrA* and *gyrB* mutations having a graded effect on DNA supercoiling (2). Using this mutant collection, we have shown that a reduction in the activity of either topoisomerase I or gyrase allows quinolone resistance to decrease. The gyrase mutations, combined with the *top*$^+$ or the *topA10* allele, might be useful for clarifying the in vivo influence of variations in the activity of topoisomerases upon the quinolone effects.

In the present study, we have analyzed the changes in plasmid DNA supercoiling following treatment with the new quinolone ciprofloxacin. Our results have revealed that in quinolone-susceptible bacteria, ciprofloxacin relaxes DNA; this effect has been observed in wild-type cells as well as in topoisomerase I and gyrase mutants. In bacteria partially resistant to quinolones, in which the gyrase activity is reduced, low levels of ciprofloxacin caused plasmid DNA supercoiling to increase whereas high levels relaxed plasmid DNA. Therefore, it is suggested that for DNA supercoiling to be increased in response to quinolone treatment, two conditions would be required: a low level of quinolone action, assumed to result from a partial resistance to the drug, and a reduction in gyrase activity. The latter condition would give rise to a DNA with a lower-than-normal level of negative superhelicity, a DNA which would then be prone to an increase in supercoiling.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* K-12 strains used, all derived from AB1157 (3), are shown in Table 1. The isolation and characterization of the *gyrA* and *gyrB* mutations have been described (2). The *gyrA904* mutation elicits a high level of quinolone resistance but has no significant effect on gyrase activity (see Fig. 2, lanes 1 and 3). The *gyrA902* mutation determines partial resistance to quinolones and originates a reduction in DNA supercoiling (see Fig. 3, lane 1). The *gyrB1521* mutation, selected for resistance to coumermycin, causes a decrease in gyrase activity (see Fig. 1, lanes 7 and 10). The susceptibility to quinolones exhibited by the *gyrA* mutants in the presence of *topA10* and *gyrB1521* mutations is presented in Table 2. Cells which grow at low quinolone levels but not at high drug concentrations are referred to as partially resistant to quinolones. Plasmid pICV90 is a Tc^s deletion derivative of pICV63, and both confer a low level of resistance to ampicillin (1). Isolation of plasmid DNA and transformation were performed as described by Maniatis et al. (14).

Media. YM9 buffer contained 11 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g of KH_2PO_4, 1 g of NH_4Cl, and 5 g of NaCl per liter. This solution, after it had been autoclaved, was supplemented

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Source or reference
IC1752[a]	*top*$^+$ *gyrB1521*	Our collection
IC2013	*top*$^+$ *gyr*$^+$	2
IC2014[b]	*topA10 gyr*$^+$	Our collection
IC2102	*topA10 gyrA902 gyrB1521*	2
IC2104	*topA10 gyrA904 gyrB1521*	2
IC2107	*top*$^+$ *gyrA904 gyrB1521*	2
IC2112	*topA10 gyrA902*	2
IC2113	*top*$^+$ *gyrA904*	2
IC2114	*topA10 gyrA904*	2
IC2703[c]	*topA10 gyrB1521*	Our collection

[a] *gyrB1521* mutation transduced into AB1157.
[b] *topA10* mutation transduced into AB1157.
[c] *gyrB1521* mutation transduced into IC2014.

with 0.1 mM $CaCl_2$ and 1 mM $MgSO_4$. YM9C medium was YM9 buffer supplemented with 400 mg of Casamino Acids (Difco), 2 g of glucose, and 2 mg of thiamine per liter.

LBT medium contained 10 g of NaCl, 10 g of Difco Bacto-Tryptone, 5 g of Difco yeast extract, and 40 mg of thymine per liter. LAT agar was LBT solidified with 2% Difco agar.

Determination of antibiotic susceptibility. Susceptibility testing was performed by a standard agar plate dilution method with serial twofold dilutions on LAT agar. The inocula were prepared by growing the strains to 10^8 cells per ml on LBT medium. The final inoculum was 10^3 CFU per spot (20 μl), and results were noted after incubation at 37°C for 24 h.

Ciprofloxacin treatment. Bacteria were grown in YM9C medium at 37°C to about 10^8 cells per ml. Ciprofloxacin (Baycip, a gift from Bayer) was added to 50-ml culture samples to the desired final concentration and then incubated at 37°C for 15 min.

Measurement of DNA supercoiling. Plasmid DNA from cells treated with ciprofloxacin and chilled just before extraction was isolated by the boiling method (14). Plasmid supercoiling was analyzed by gel electrophoresis in agarose gels containing chloroquine by the method of Pruss (16), with modifications described previously (1).

RESULTS

Ciprofloxacin relaxes plasmid DNA in quinolone-susceptible cells. The effects of ciprofloxacin treatment in quinolone-susceptible cells are shown in Fig. 1. Ciprofloxacin caused plasmid pICV90 DNA to relax in strain IC2013 (*top*$^+$ *gyr*$^+$) (lanes 1 through 3), as well as in IC2014 (*topA10 gyr*$^+$) (lanes 4 through 6). Moreover, since the deficiency in topoisomerase I causes a more negatively supercoiled plasmid DNA (compare lanes 1 and 4), the change in supercoiling promoted by ciprofloxacin in *topA10* mutants appears to be more important than in *top*$^+$ cells.

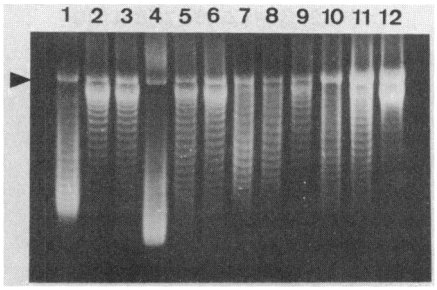

FIG. 1. Gel electrophoresis assay of pICV90 DNA isolated from quinolone-susceptible cells treated with increasing concentrations of ciprofloxacin. Plasmid DNA isolated from equal-volume cultures was run on an 0.8% agarose gel containing 10 μg of chloroquine per ml. The direction of migration was from top to bottom. Under the conditions used, some samples are in part positively supercoiled (lanes 2, 3, 5, 6, 9, 11, and 12) and the more negatively supercoiled plasmids migrate more rapidly. For each strain, ciprofloxacin concentrations assayed were (from left to right) 0, 0.5, and 1 μg/ml. Arrowhead, Relaxed-nicked DNA. Lanes 1 through 3, *top*$^+$ *gyr*$^+$ (IC2013); lanes 4 through 6, *topA10 gyr*$^+$ (IC2014); lanes 7 through 9, *top*$^+$ *gyrB1521* (IC1752); lanes 10 through 12, *topA10 gyrB1521* (IC2703).

In bacteria carrying the *gyrB1521* mutation, either *top*$^+$ (IC1752) or *topA10* (IC2703), the relaxing effect of ciprofloxacin on plasmid DNA was less than in *gyr*$^+$ cells (Fig. 1, lanes 7 through 12). This could be due to the fact that in *gyrB1521* mutants, plasmid DNA is partially relaxed, and it would be difficult for it to be made still more relaxed by the action of the quinolone. Note that under the experimental conditions used, positively supercoiled DNA was more apparent in *topA10 gyrB1521* cells than in *top*$^+$ *gyrB1521* bacteria after treatment with ciprofloxacin (Fig. 1, compare lanes 9 and 12).

Ciprofloxacin effects in cells partially resistant to quinolones. The DNA relaxation effect of ciprofloxacin observed in quinolone-susceptible cells would be expected given that quinolones interfere with the activity of DNA gyrase. However, it has been reported that treatment of the triple mutant GP200 (Δ*topA gyrA307 gyrB225*), which exhibits a partial resistance to quinolones, with oxolinic acid causes chromosomal as well as plasmid DNA supercoiling to increase (12, 17). Since the dependence of this increase on the different topoisomerase mutations present in GP200 cells is not known, we wished to clarify this dependence by using a collection of isogenic strains carrying the *top*$^+$ or the *topA10* allele combined with *gyrA* and *gyrB* mutations. These strains exhibited differences in both susceptibility to quinolones (Table 2) and gyrase activity.

TABLE 2. Susceptibility of *gyrA* mutants to quinolones

Strain	Relevant genotype	MIC (μg/ml)[a]	
		Nalidixic acid	Ciprofloxacin
IC2113	*top*$^+$ *gyrA904*	600	0.4
IC2107	*top*$^+$ *gyrA904 gyrB1521*	300	0.4
IC2114	*topA10 gyrA904*	200	0.3
IC2104	*topA10 gyrA904 gyrB1521*	100	0.2
IC2112	*topA10 gyrA902*	50	0.1
IC2102	*topA10 gyrA902 gyrB1521*	20	0.1
IC2013	*top*$^+$ *gyr*$^+$	4	0.03

[a] Concentration inhibiting colony formation by more than 90%.

First, we treated strain IC2104 (*topA10 gyrA904 gyrB1521*), which carries two well-characterized gyrase mutations, with the quinolone ciprofloxacin and then examined the variations in the supercoiling of the reporter plasmid pICV90. In IC2104, the *gyrB1521* mutation causes a decrease in both DNA supercoiling and quinolone resistance promoted by the *gyrA904* mutation (Table 2).

Plasmid DNA became more negatively supercoiled after treatment of strain IC2104 with ciprofloxacin, the increase in

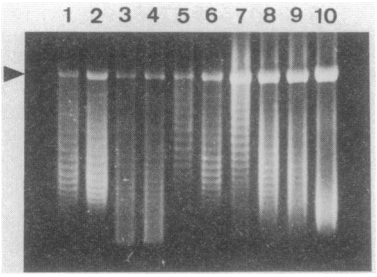

FIG. 2. Effect of ciprofloxacin treatment on strains carrying the *gyrA904* allele. pICV90 DNA was isolated from equal-volume cultures incubated with increasing concentrations of ciprofloxacin, and electrophoresis was carried out with an 0.8% agarose gel containing 10 μg of chloroquine per ml. At this chloroquine concentration, all supercoiled species were negatively supercoiled. Ciprofloxacin concentrations were 0 (lanes 1, 3, 5, and 7), 0.5 (lanes 2, 4, 6, and 8), 1 (lane 9), and 3 (lane 10) μg/ml. Strains other than IC2104 displayed a similar pattern of plasmid supercoiling for ciprofloxacin concentrations from 0.5 to 3 μg/ml. Arrowhead, Relaxed-nicked DNA. Lanes 1 and 2, *top*$^+$ *gyrA904* (IC2113); lanes 3 and 4, *topA10 gyrA904* (IC2114); lanes 5 and 6, *top*$^+$ *gyrA904 gyrB1521* (IC2107); lanes 7 through 10, *topA10 gyrA904 gyrB1521* (IC2104).

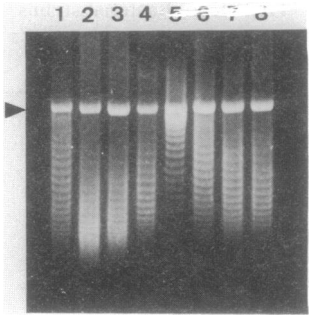

FIG. 3. Gel electrophoresis assay of plasmid DNA isolated from strains carrying the *gyrA902* allele. Plasmid pICV90 isolated from cultures treated at increasing concentrations of ciprofloxacin was run on an 0.8% agarose gel containing 12.5 μg of chloroquine per ml. Under the conditions used, most of the supercoiled species are negatively supercoiled, and the more negatively supercoiled plasmids migrate more rapidly. For each strain, ciprofloxacin concentrations assayed were (from left to right) 0, 0.5, 1, and 3 μg/ml. Arrowhead, Relaxed-nicked DNA. Lanes 1 through 4, *topA10 gyrA902* (IC2112); lanes 5 through 8, *topA10 gyrA902 gyrB1521* (IC2102).

supercoiling being greater at high quinolone levels than at low levels (Fig. 2, lanes 7 through 10). This result was therefore similar to that found by Franco and Drlica (12) when strain GP200 was treated with oxolinic acid. Also shown in Fig. 2 are the effects of ciprofloxacin on plasmid DNA occurring in *gyrA904* cells which carried the *topA10* or the *gyrB1521* mutation alone. Supercoiling clearly increased in IC2107 (*top*$^+$ *gyrA904 gyrB1521*) treated with ciprofloxacin (lanes 5 and 6) although to a lesser degree than in the triple mutant IC2104 (*topA10 gyrA904 gyrB1521*) (compare lanes 6 and 8). In *gyrA904 gyrB*$^+$ cells, either *top*$^+$ (IC2113; lanes 1 and 2) or *topA10* (IC2114; lanes 3 and 4), ciprofloxacin had no significant effect on plasmid DNA supercoiling.

The results indicate that transition of DNA to higher supercoiling promoted by ciprofloxacin appeared to depend on the presence of two gyrase mutations. In our cells, the *gyrA904* allele conferred a high level of quinolone resistance but did not cause a significant gyrase deficiency. Thus, in order to make possible the increase in supercoiling, a second gyrase mutation such as *gyrB1521*, which determines both a reduction in DNA supercoiling and a decrease in the GyrA-dependent quinolone resistance, might be needed. In contrast, the increase in supercoiling would not depend on topoisomerase I deficiency.

The requirement for two gyrase mutations might be dispensable in cells carrying a gyrase mutation able to confer partial resistance to quinolones and originating a reduction in DNA supercoiling. We have isolated this class of mutations (2) and have decided to examine their influence on ciprofloxacin action.

The results obtained with cells carrying the *gyrA902* mutation (see Materials and Methods and reference 2) are shown in Fig. 3. The cells also carried the *topA10* mutation, since the variations in supercoiling were better observed when topoisomerase I was deficient. In IC2112 (*topA10 gyrA902*), supercoiling increased at low ciprofloxacin levels (Fig. 3, lanes 1 and 2), which confirms our prediction about the possibility of increasing supercoiling in the presence of only one gyrase mutation, affecting both quinolone resistance and gyrase activity. Surprisingly, when the quinolone concentration was increased, DNA appeared to relax (Fig. 3, lanes 3 and 4), suggesting that ciprofloxacin was acting as it did in drug-susceptible cells. In the triple mutant IC2102 (*topA10 gyrA902 gyrB1521*), the pattern was similar to that seen in IC2112 (Fig. 3, lanes 5 through 8).

DISCUSSION

In this paper we have shown that treatment of quinolone-susceptible *E. coli* cells with ciprofloxacin causes relaxation of plasmid DNA. This relaxation occurs even in *topA10* mutants having little topoisomerase I activity (7). Therefore, it seems that in the presence of ciprofloxacin, other sources of relaxing activity, such as gyrase itself or topoisomerase III (6), are acting in the cell.

The result observed with the *topA10* mutants is in contrast with that of Bliska and Cozzarelli (4), who observed no plasmid DNA relaxation after treatment of *topA* mutants with norfloxacin. This apparent discrepancy could be due to either the antibiotic tested or the DNA examined. In our strains, a relaxing effect of norfloxacin similar to that found with ciprofloxacin was observed (data not shown). Moreover, the results were also similar whether plasmid pICV90 or the unrelated plasmid pACYC184 (5) was used (data not shown).

Ciprofloxacin increases negative superhelicity of plasmid DNA in strain IC2104 (*topA10 gyrA904 gyrB1521*), which is partially resistant to quinolones. This finding confirms the result observed by Franco and Drlica (12) after oxolinic acid treatment of strain GP200 (Δ*topA gyrA307 gyrB225*). However, we have demonstrated that neither the topoisomerase I mutation nor the combination of two gyrase mutations is essential to elicit a ciprofloxacin-promoted increase in supercoiling of plasmid DNA. Further, we have shown that with a single strain such as IC2112 (*topA10 gyrA902*) (Fig. 3, lanes 1 through 4), it was possible to observe two opposite effects of ciprofloxacin, that is, an increase in supercoiling at low levels and relaxation at higher levels. Therefore, we think that the conditions necessary for an increase in plasmid supercoiling in response to ciprofloxacin treatment would be a low level of quinolone action, due to partial drug resistance, and a DNA in a steady state, with lower-than-normal

negative superhelicity. The low level of ciprofloxacin action could transiently inhibit the supercoiling activity of gyrase, thus relaxing the DNA to a point at which transition to a more negatively supercoiled DNA would be triggered. As the quinolone concentration is raised and gyrase activity becomes more inhibited, further supercoiling would be prevented and DNA would then appear to relax, as happens in quinolone-susceptible cells. According to this explanation, the response to gyrase inhibitors would involve homeostatic regulation of supercoiling dependent on changes in the expression of the gyrase genes (15). This possibility is consistent with the results of Franco and Drlica (12), which suggest that the increase in DNA supercoiling promoted by quinolones is due to an increased rate of expression of the *gyrA* gene.

An increase in plasmid supercoiling can result from the transcription of plasmid genes (21). Therefore, transcription could mediate the effects on plasmid supercoiling of quinolone treatment in topoisomerase mutants. This possibility is not supported by the results of Franco and Drlica (12), which indicate that the levels of supercoiling are similar in spite of variations on transcription from a plasmid promoter in the absence of quinolone treatment. In any case, it would not change the validity of our conclusion regarding the requirements for the increase in DNA supercoiling promoted by quinolones.

Our results have shown that both bacterial mutations which affect DNA supercoiling and plasmids as probes of DNA topology are useful elements for studying the in vivo response to gyrase inhibitors. These elements might also serve in the analysis of other changes in supercoiling, such as those involved in the adaptation of bacteria to the stress resulting from environmental conditions.

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