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Association between Early Inhibition of DNA Synthesis and the MICs and MBCs of Carboxyquinolone Antimicrobial Agents for Wild-Type and Mutant [*gyrA nfxB(ompF) acrA*] *Escherichia coli* K-12

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Quinolone antimicrobial agents are known to interact with DNA gyrase, but the mechanism by which bacterial cell death occurs is not fully understood. In order to determine whether there is a correlation between quinolone-induced inhibition of early (i.e., 10 to 15 min) DNA synthesis and potency (MICs and MBCs), we measured the rate of DNA synthesis in log-phase *Escherichia coli* K-12 by using [^{3}H]thymidine incorporation. Three quinolones (ciprofloxacin, norfloxacin, and difloxacin) were selected based on their decreasing activity against reference strain KL16. All three quinolones caused an early 50% inhibition of DNA synthesis which was proportional to MICs and MBCs ($r > 0.99$). Furthermore, 50% inhibition of DNA synthesis and MICs were nearly identical for mutant strains with an altered quinolone target (*gyrA*) or with decreased [*nfxB(ompF)*] or increased (*acrA*) permeability. There were significant differences ($P < 0.001$) between individual quinolones in the degree of DNA synthesis inhibition in nalidixic acid-resistant *gyrA* and *nfxB(ompF)* mutant strains. The comparison of the three mutants with the wild-type strain permitted an in vivo examination of the effects of alterations of the drug target or entry on the activity determined by DNA synthesis inhibition and MICs.

The mechanism(s) by which quinolone antimicrobial agents kill microorganisms is unknown (4, 7, 20, 31, 38). Clinically relevant concentrations of quinolones or naphthyridines such as norfloxacin, ciprofloxacin, oxolinic acid, and nalidixic acid inhibit DNA rather than protein or RNA synthesis. It is believed that antimicrobial agent-induced effects on bacterial DNA topoisomerase II (gyrase) trigger a cascade of events that results in a rapid loss of cell viability (7, 20, 31). The quinolone antimicrobial agents perturb several gyrase-mediated functions, including supercoiling, decatenation, and unknotting (35, 36, 38). Since bacterial but not eucaryotic topoisomerases supercoil DNA in vitro (35), the specificity of these antimicrobial agents for procaryotes has been attributed to carboxyquinolone-induced cytotoxicity associated with the inhibition of supercoiling (20, 31, 38). However, results of in vitro studies in *Escherichia coli* indicate that while the inhibition of supercoiling bears a relationship with the MICs (2, 20, 22, 29, 30), the concentrations required to inhibit supercoiling in extracts from wild-type or *gyrA* mutants of *E. coli* by 50% (IC_{50}s) are generally 1 to 2 orders of magnitude greater than the corresponding MICs (2, 6, 20, 22, 29, 30). Moreover, concentrations which inhibit supercoiling in vitro are the same as those required in vivo (both are several orders of magnitude greater than the corresponding MIC [12, 32]), and there is a poor correlation between supercoiling IC_{50}s and MICs for gram-positive bacteria (39). Since quinolone uptake is not energy dependent in bacteria (2), active uptake associated with high intracellular drug concentrations is not a likely explanation for the quantitative discrepancies between supercoiling IC_{50}s and MICs.

In previous studies in which the antibacterial effects of quinolones on gyrase function were examined, the in vitro inhibition of supercoiling or sodium dodecyl sulfate-induced DNA breakage with MICs was compared (6, 20, 29). However, based on elegant in vivo studies by Drlica and colleagues (8–11, 32), in which the mechanism of action of oxolinic acid was examined, we hypothesized that early, gyrase-mediated, supercoiling-independent inhibition of DNA synthesis is the initial event that leads to cell death. Drlica and colleagues (8–11, 32) have demonstrated that in *recA* and *lexA* mutants (cells which cannot induce SOS repair), there are two phases of inhibition, a rapid initial phase which appears to be proportional to the drug concentration and a second, slower phase which is absent in *recA*$^+$ (wild-type) cells (9, 11, 32). Both phases of DNA synthesis inhibition occur in the absence of decreased superhelical density (i.e., at concentrations which do not inhibit supercoiling in vivo). Moreover, the initial phase is proportional to the drug concentrations (32) and is not decreased by the addition of chloramphenicol or rifampin (9), antibiotics which inhibit both SOS-related DNA repair processes (9) and carboxyquinolone-induced lethal effects (4, 20).

In order to determine whether there is a correlation between quinonlone-induced early inhibition of in vivo DNA synthesis and the subsequent inhibitory and lethal events, we examined the association between the IC_{50}s for DNA synthesis in vivo and the MICs and MBCs for several quinolones. These studies were performed in K-12, a well-characterized wild-type strain of *E. coli* (4, 5, 21, 28, 31, 33), as well as in several mutants, including two nalidixic acid-resistant [*gyrA* and *nfxB(ompF)*] mutants (20, 21) and a

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hypersusceptible *acrA* mutant which is known to have a defect in outer membrane barrier function (3).

MATERIALS AND METHODS

Chemicals and reagents. Ciprofloxacin was a gift from Miles Pharmaceuticals (West Haven, Conn.), norfloxacin was a gift from Merck Sharp & Dohme (West Point, Pa.), and difloxacin was a gift from Abbott Laboratories (North Chicago, Ill.). All antimicrobial agents were filter sterilized, and portions were stored at −20°C in distilled water or 0.02 N NaOH (norfloxacin). Determinations of MICs, MBCs, and IC_{50}s were performed in log-phase cells (determined turbidimetrically at 600 nm) in M9 broth supplemented with thiamine (0.1 μg/ml) and adjusted to pH 7.5 with potassium phosphate buffer (final concentration, 100 mM) and sodium bicarbonate (final concentration, 10 mM). Broth macrodilutions of MICs and MBCs were performed in modified M9 medium with an inoculum of 5×10^5 CFU/ml, according to standard techniques (26). The MIC was defined as the lowest concentration that inhibited visible growth after 18 to 24 h of incubation at 37°C. MBCs were defined as the lowest concentrations of antimicrobial agents associated with >99.9% killing of the original inoculum after a subsequent 18- to 24-h incubation on chocolate agar.

Bacterial strains. The following *E. coli* K-12 strains were used: KL16 (wild type) (4, 5, 21, 28, 31, 32); KF130, a *gyrA* mutant of KL16 (20, 21); KF131, a *nfxB*(*ompF*) mutant derivative of KL16 (20, 22); and CL2, an *acrA* derivative mutant (3). Strains KL16, KF130, and KF131 were gifts from D. Hooper; and strain CL2 was a gift from W. Coleman.

Measurement of DNA synthesis. The rate of DNA synthesis in log-phase cells was determined in modified M9 broth by using the technique described by Drlica et al. (9). Briefly, 200 μl of exponentially growing cells was pulse labeled by transferring portions into borosilicate tubes containing 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.). The cells were incubated for 2 min, and the incorporation of radioactivity was terminated by adding 2 ml of cold 5% trichloroacetic acid. Precipitates were collected by filtration through GF/C filters (Whatman, Inc., Clifton, N.J.), which were then washed with 5% trichloroacetic acid and 95% ethanol. Acid-precipitable radioactivity was taken as a measurement of the rate of DNA synthesis, as measured by liquid scintillation counting.

The kinetics of DNA synthesis inhibition were measured with 2-min pulses of thymidine by using several concentrations of each of three carboxyquinolones over 30 min. In order to determine the appropriate concentrations for subsequent studies, dose-response curves were generated with a wide range of drug concentrations. In subsequent experiments a control flask (no antibiotic), a 100% inhibition flask, and four flasks with concentrations which were previously shown to fall in the linear portion of the curve were included. DNA synthesis was measured at both 10 and 15 min of incubation, and the time point showing the maximal inhibition was used to construct dose-response curves.

Statistical methods. For each drug-strain combination, at least three different experiments were performed; Fig. 1 through 3 consist of composite data from all experiments. The concentrations that caused maximal inhibition and the concentrations associated with 15 to 85% inhibition, the linear portion of sigmoidal curves, were determined; and best-fit lines were calculated for all points between 15 and 85% of DNA synthesis inhibition by using least-squares analysis (37). IC_{50}s were then determined from the best-fit lines and compared with MICs and MBCs. Because of the induction of DNA repair, maximal (i.e., 100%) inhibition did not correspond to complete inhibition of DNA synthesis. To provide the statistical power necessary to compare the effects of individual antibiotics on mutant strains, linear models were also constructed by using the Glim program (The Glim System Release 3.77, Royal Statistical Society, Numerical Algorithms Group, Downers Grove, Ill., 1986) with three variables, including drug, log concentration, and strain, and appropriate interaction terms. The significance of each variable in our model was tested by using the standard F-test criteria for reduction in the error of the sum of squares (37).

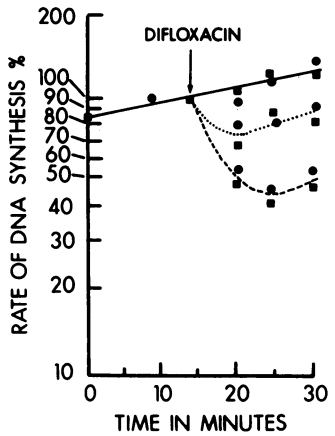

FIG. 1. Inhibition of DNA synthesis by difloxacin in the wild-type strain KL16 (■) (*recA*$^+$) and the quinolone target mutant strain KF130 (●) (*recA*$^+$ *gyrA*$^-$). The arrow indicates the time of difloxacin addition. The solid line indicates the rate of DNA synthesis in the untreated control, the dotted line indicates a difloxacin concentration equal to the MIC, and the dashed line indicates a difloxacin concentration that is 4 times the MIC.

RESULTS

In order to demonstrate that *recA*$^+$ (i.e., wild-type) strains exposed to quinolones have an initial decay in the rate of DNA synthesis followed by repair (9, 11, 32), the effects of quinolones on DNA synthesis were measured. DNA synthesis rates in strains KL16 and KF130 (*gyrA*) with or without difloxacin are shown in Fig. 1. The concentrations of difloxacin used in this experiment were 0.25 and 1.0 μg/ml for KL16 and 8.0 and 32 μg/ml for KF130, corresponding to concentrations equal to or 4 times the MICs, respectively (Table 1). As originally described by Drlica (9), after a nadir in the rate of DNA synthesis occurred, cellular repair mechanisms responded to the drug-induced damage with an increase in DNA synthesis.

TABLE 1. Comparison of IC_{50}s and MICs in wild-type and mutant strains

Strain	Ciprofloxacin		Norfloxacin		Difloxacin	
	IC_{50} (μg/ml)	MIC (μg/ml)	IC_{50} (μg/ml)	MIC (μg/ml)	IC_{50} (μg/ml)	MIC (μg/ml)
KL16	0.014	0.01	0.09	0.03	0.25	0.25
KF130	0.64	0.50	1.70	1.0	8.8	8.0
KF131	0.025	0.015	0.29	0.25	0.26	0.25

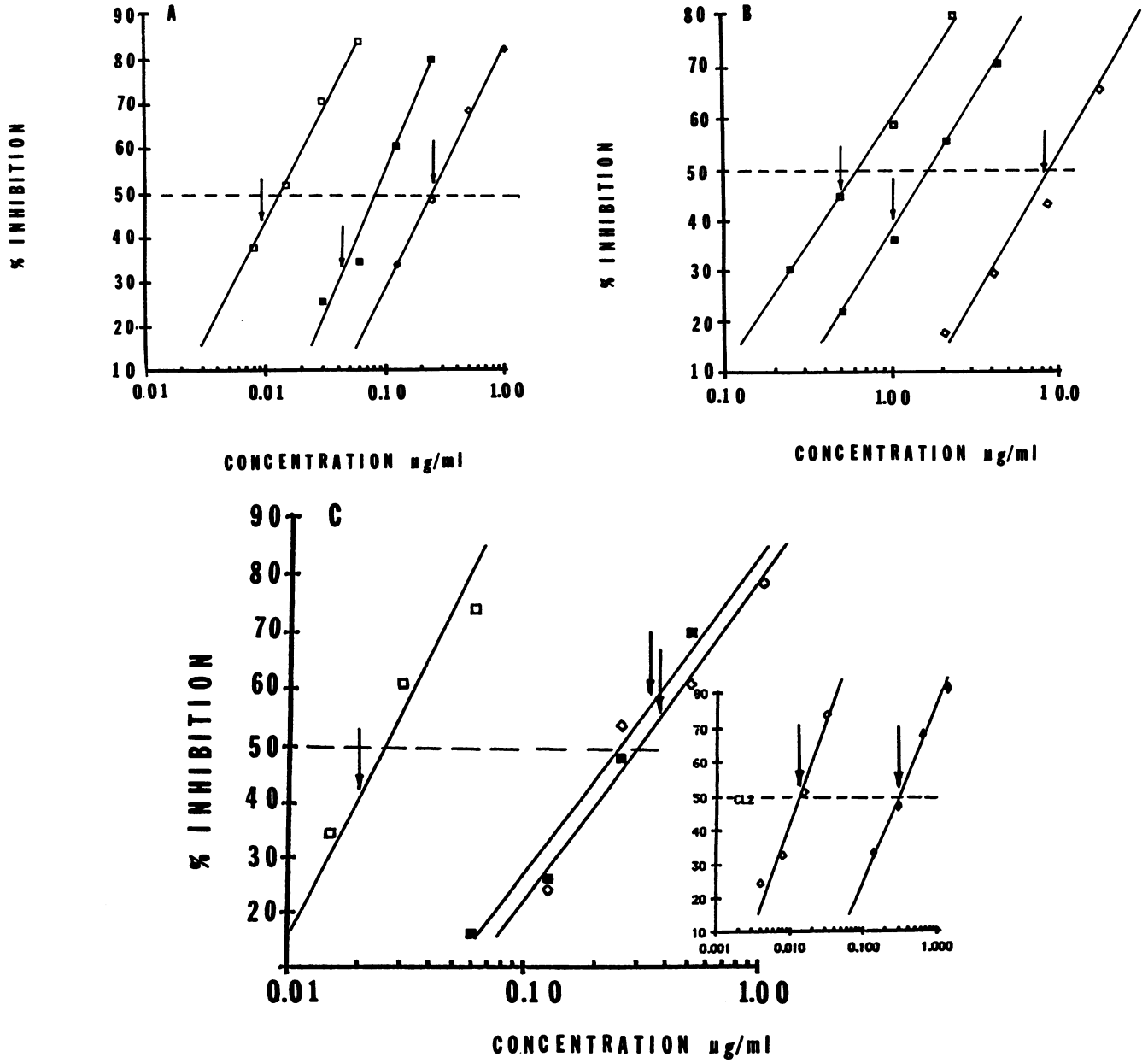

FIG. 2. Percent inhibition of DNA synthesis in *E. coli* KL16, KF130, KF131, and CL2. The solid lines represent the best fit of composite data from points falling between 15 and 85% inhibition. The hatched line intersects the IC_{50}, and the arrows show the MIC of each compound. (A) Percent inhibition of DNA synthesis in KL16 for ciprofloxacin (□; $r = 0.94$), norfloxacin (■; $r = 0.92$), and difloxacin (◇; $r = 0.95$). (B) Percent inhibition of DNA synthesis in KF 130, the *gyrA* mutant, for ciprofloxacin (□; $r = 0.88$), norfloxacin (■; $r = 0.85$), and difloxacin (◇; $r = 0.95$). (C) Percent inhibition of DNA synthesis in KF131, the *nfxB*(*ompF*) mutant, by ciprofloxacin (□; $r = 0.91$), norfloxacin (■; $r = 0.85$), and difloxacin (◇; $r = 0.94$). The inset shows the percent inhibition of DNA synthesis by difloxacin for KL16, the wild-type strain (◆; $r = 0.98$), and CL2, the *acrA* mutant (◇; $r = 0.97$).

The association between the nadir of early DNA synthesis inhibition with several concentrations of three quinolones is shown in Fig. 2A for wild-type *E. coli* KL16. The IC_{50}s of each drug which inhibited early synthesis were calculated from the best-fit lines, which described the linear portion of the sigmoidal dose-response curves (i.e., 15 to 85% inhibition). These are given in Table 1 along with the corresponding MICs. MICs and MBCs were identical for all strains (KL16, KF130, KF131, CL2) and drugs (ciprofloxacin, norfloxacin, and difloxacin; data not shown). The most active quinolone, ciprofloxacin, had an IC_{50} of 0.014 μg/ml and a MIC of 0.01 μg/ml (Fig. 2A and Table 1). The dose-response curve for the next most active drug, norfloxacin, was shifted to the right and had a MIC of 0.03 μg/ml and a IC_{50} of 0.09 μg/ml. The correlation between the IC_{50} and the MIC for the least-active drug, difloxacin, was also excellent, with identical values of 0.25 μg/ml. Results of preliminary studies (data not shown) with *Staphylococcus aureus* 86 (25) also suggested that there is a good correlation between the IC_{50} for DNA synthesis inhibition MICs; for ciprofloxacin the IC_{50} was 0.67 μg/ml and the MIC was 1.0 μg/ml.

The dose-response effects on early DNA synthesis inhibi-

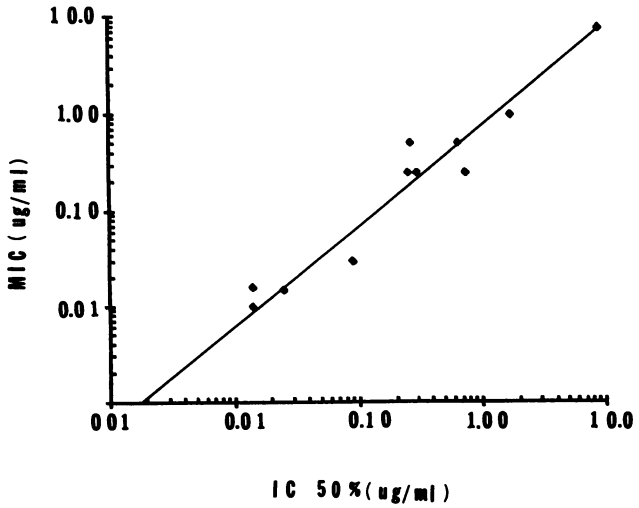

FIG. 3. MICs for all *E. coli* strains (KL16, KF130, KF131, and CL2) and compounds (ciprofloxacin, norfloxacin, and difloxacin) plotted with the corresponding IC_{50}s ($r > 0.99$).

tion for the quinolone antimicrobial agents in mutant strains are shown in Fig. 2B and C. KF130 *gyrA* and KF131 *nfxB*(*ompF*) are transductants of KL16 (wild type) and, therefore, are isogenic (21). Dose-response DNA inhibition studies were performed in these strains to determine whether the correlation between the IC_{50} and MICs held for strains which either had an altered drug target or a permeability defect. Moreover, we wanted to determine whether the relative resistance associated with each mutation differed with individual quinolone antibiotics.

The IC_{50}s and the MICs in the *gyrA* (antimicrobial agent target) mutant were increased (Fig. 2B and Table 1). The excellent correlation between MICs and IC_{50}s remained for all drugs. The rank order of quinolone activity, as measured by either IC_{50}s or MICs, was the same as that for KL16 (compare Fig. 2A and B). On the other hand, the relative increase in IC_{50}s and MICs differed for individual antimicrobial agents. Ciprofloxacin changed the least; the IC_{50} rose 19-fold and the MIC rose 32-fold. Norfloxacin showed the greatest increase; the IC_{50} rose 46-fold and the MIC rose 50-fold. Difloxacin had an increase that was intermediate between those of ciprofloxacin and norfloxacin. The differences in the relative increase in IC_{50}s between individual antimicrobial agents were highly significant ($P < 0.001$).

Quinolone dose-response effects on DNA synthesis inhibition in the *ompF* (permeability) mutant KL131 are shown in Fig. 2C. These mutants show decreased susceptibility to quinolones (17, 18, 21), which is associated with a decreased uptake (17). Data for KF131 again demonstrated an excellent correlation between the MICs and IC_{50}s and the rank order of activity for all drugs. However, each drug again demonstrated a different degree of change in the MICs and inhibition of DNA synthesis compared with that of wild-type *E. coli*. In KF131 difloxacin showed no increase in either the IC_{50} or the MIC when compared with those of KL16. Both ciprofloxacin and norfloxacin were significantly less active (i.e., greater IC_{50}s) in KF131 than in KL16 ($P < 0.001$). Additionally, in KF131 both antimicrobial agents demonstrated a greater increase in IC_{50}s than did difloxacin ($P < 0.001$). While norfloxacin was less active than ciprofloxacin in this mutant strain, this difference did not quite reach a level of significance ($P = 0.06$; $n = 51$). The inset to Fig. 2C shows the difloxacin dose-response effects on DNA synthesis inhibition in wild-type strain KL16 and *acrA* mutant CL2. This *acrA* mutant is also a K-12 derivative and possesses a leaky outer membrane phenotype (3, 19, 24). In contrast to *ompF* mutants, these mutants demonstrate increased permeability to a variety of chemicals and antibiotics (3, 24; unpublished data) as well as increased susceptibility to quinolones (3) and novobiocin (3, 14). The MICs of carboxyquinolones in CL2 decreased by 2- to 16-fold; the greatest change was for difloxacin, and the least change was for norfloxacin. A comparison of the dose-response curves for KL16 and CL2 showed that the latter was significantly ($P < 0.001$) shifted to the left; that is, a lower drug concentration inhibited DNA synthesis when compared with the wild-type strain. In CL2 the IC_{50} and MIC of difloxacin were 0.014 and 0.015 μg/ml, respectively, whereas both were 0.25 μg/ml in KL16.

These data were analyzed statistically by using the Glim computer program, as described above. The standard F test was used to compare the improvement in the model with each additional variable. Using this analysis, we demonstrated that the log drug concentration, drug type (ciprofloxacin, norfloxacin, or difloxacin), and mutation status [wild type, *gyrA*, *nfxB*(*ompF*), *acrA*] were all significant in the linear model, with P values of <0.001 for each.

The association between the MICs and the IC_{50}s for all three quinolones in all strains tested is shown in Fig. 3. Regardless of the drug or mutant status, there was an excellent correlation ($r > 0.99$) between MICs and IC_{50}s. Notably, the slope was 1 and the line passed near the origin.

DISCUSSION

Results of studies by other investigators have demonstrated that low concentrations of carboxyquinolones inhibit DNA synthesis (4, 7, 8–11, 15, 32) rather than protein or RNA synthesis (4, 7, 15). However, with the exception of oxolinic acid (32), there are no data for the dose-response kinetics of early DNA synthesis inhibition as it relates to antimicrobial activity, and DNA synthesis inhibition is examined in most studies after the initial nadir is reached. Since wild-type strains (but not *recA* or *lexA* mutants) show a poor correlation between early and late DNA synthesis inhibition because of SOS-induced DNA repair (9), we compared early DNA synthesis inhibition with the MICs. In a previous study (9) it has been shown that the initial rapid phase of inhibition of DNA synthesis is identical in wild-type and *recA* strains.

We studied several well-characterized *E. coli* strains: a K-12 wild-type strain (KL16) and mutants which differed either in the putative quinolone lethal target (DNA gyrase subunit A [13, 16, 23, 34], strain KF130) or in outer membrane barrier function. The comparison of the three classes of mutants with the wild-type strain permitted an in vivo examination of the effects of alterations in either drug target or entry on drug activity, as determined by DNA synthesis inhibition and MICs. Moreover, the increased activity of carboxyquinolones, particularly difloxacin, in the *acrA* mutant suggests that the outer membrane of wild-type (*ompF*$^+$) *E. coli* is a barrier to the uptake of these compounds, as has been suggested by others (17).

Data showing that the relative decrease in activity (IC_{50}s and MICs) for individual quinolones differs in *gyrA* and *nfxB*(*ompF*) mutants may be useful in developing strategies to circumvent the development of resistance while a patient is receiving drug therapy. The mutant strains that we studied

were resistant to nalidixic acid and showed very high IC_{50}s for DNA synthesis inhibition by this agent (data not shown). In contrast, resistance to norfloxacin in KL16 requires at least two mutations [*gyrA nfxB*(*ompF*)] (21). Resistance caused by two mutations occurs very infrequently (1). However, it is possible that some patients may already harbor *ompF* mutant strains, since this mutation decreases susceptibility not only to quinolones but also to other classes of antibiotics, including β-lactams (27). A comparison of the relative activities of several fluoroquinolone antibiotics in double mutants would be of both clinical and theoretical importance.

Results of this study indicate that there is an excellent correlation ($r > 0.99$) between the early inhibition of DNA synthesis (i.e., prior to the induction of SOS repair) and the corresponding MICs for all strains and carboxyquinolones tested. In contrast to studies in which the IC_{50}s for supercoiling and MICs have been compared, the IC_{50}s for DNA synthesis and MICs were nearly identical. Results of this study are consistent with our hypothesis that the initial event in the cascade leading to carboxyquinolone-induced cell death may be gyrase-mediated (9), supercoiling-independent early inhibition of DNA synthesis. Inhibitors of protein (chloramphenicol) or RNA (rifampin) synthesis prevent killing but not the quinolone-induced early inhibition of DNA synthesis (9). This suggests that SOS repair processes resulting from quinolone-induced DNA damage may be associated with cell death. A similar hypothesis has also recently been proposed by Drlica (personal communication; K. Drlica and R. J. Franco, manuscript in preparation) and Diver and associates (J. M. Diver, L. J. V. Piddock, and R. Wise, Int. Congr. Chemother. abstr. no. 985, Istanbul, Turkey, 1987).

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