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Novel Quinolone Resistance Mutations of the *Escherichia coli* DNA Gyrase A Protein: Enzymatic Analysis of the Mutant Proteins

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Using the techniques of gap misrepair mutagenesis and site-directed mutagenesis, we have generated two novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein. DNA sequencing showed these mutations to be Ser-83→Ala and Gln-106→Arg. The mutant proteins were overproduced and purified, and their enzymatic properties were analyzed and compared with those of the wild-type enzyme. With ciprofloxacin and other quinolones, the inhibition of DNA supercoiling, relaxation, and decatenation and the induction of DNA cleavage were investigated for both wild-type and mutant enzymes. In each assay, the mutant enzymes were found to require approximately 10 times more drug to inhibit the reaction or induce cleavage than was the wild-type enzyme. However, the Ca^{2+}-directed DNA cleavage reaction was indistinguishable for wild-type and mutant gyrases. We discuss models for the gyrase-mediated bactericidal effects of quinolone drugs.

The quinolones are a clinically useful group of antibacterial agents. Since the synthesis of the progenitor, nalidixic acid (NAL) (20), a large number of derivatives have been synthesized and evaluated for their antibacterial activities (29, 42). Among the most potent of these drugs are the fluoroquinolones, such as norfloxacin (NFX) and ciprofloxacin (CFX), which are established therapeutic agents (28). The molecular target for the quinolone drugs is thought to be DNA gyrase (8), the bacterial enzyme which supercoils DNA by using the free energy of ATP hydrolysis (for reviews, see references 7 and 41).

DNA gyrase from *Escherichia coli* consists of two proteins, A and B, with molecular masses of 97 and 90 kDa, respectively. The active enzyme is an A_2B_2 complex (16, 18). The genes for the gyrase proteins, *gyrA* and *gyrB*, have been sequenced and cloned such that the A and B proteins can be overproduced as up to 40% soluble cell proteins (1, 9, 14, 15, 25, 39, 43, 46). The mechanism of action of DNA gyrase involves the ATP-driven passage of a segment of DNA through a double-strand DNA break, stabilized in part by covalent bonds between the DNA and the protein (reviewed in reference 23). The presence of quinolone drugs inhibits the supercoiling, relaxation, and decatenation reactions performed by gyrase in vitro (8, 23). All of these reactions require the breakage and reunion of DNA. Incubation of gyrase and DNA in the presence of a quinolone drug and termination of the reaction by the addition of sodium dodecyl sulfate (SDS) lead to the breakage of the DNA in both strands. Moreover, the A subunit is found to be covalently bound to the newly formed 5′-phosphate at the break site via a phosphotyrosine bond to Tyr-122 (14). By contrast, very little cleavage is detected with gyrase isolated from a quinolone-resistant strain (8). These observations provide support for the A subunit being involved in the breakage and reunion of DNA by gyrase and being the likely site of action of the quinolone drugs. That quinolone resistance mutations map to *gyrA* also supports the contention that this subunit is the site of action of these drugs (reviewed in references 29 and 42).

Despite the wealth of information about DNA gyrase and the quinolone drugs, the mode of action of the drugs is not known; indeed, their primary site of action is still a contentious issue. Although quinolone resistance mutations have been mapped to *gyrA*, mutations have been mapped to other loci, including *gyrB* (43, 44). A recent report showed that among 25 spontaneous quinolone-resistant mutants, 13 had *gyrA* mutations and 12 had *gyrB* mutations (26). In addition, there is evidence that the drugs bind primarily to DNA rather than to the enzyme (36). Further binding data suggest that quinolones bind to the gyrase-DNA complex (34), and a model in which the drugs interact with both gyrase and single-stranded DNA revealed at the DNA cleavage site in the active site of the enzyme has been proposed (35).

A further unresolved question concerning the quinolone drugs is the manner in which they exert their bactericidal and bacteriostatic effects. This is manifested by the lack of correlation between MICs and either the concentrations of drug required to inhibit the gyrase supercoiling reaction in vitro by 50% or the concentrations of drug required to induce DNA cleavage in vitro (8, 48). Typically, the concentrations of drug required to inhibit supercoiling or elicit DNA cleavage exceed MICs by 10- to 100-fold, implying that the inhibition of DNA supercoiling or the formation of the cleavable complex by the drugs is not necessarily a requirement for their bactericidal activity.

One approach to studying the action of quinolone drugs is to analyze the properties of quinolone-resistant gyrase proteins. We have generated quinolone resistance mutations of the gyrase A (GyrA) protein by two different methods and have analyzed some of the reactions of the purified proteins in vitro, with a view to elucidating the mechanism of action of the quinolones.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study are derivatives of *E. coli* K-12. Strain JM109 [*recA1 hsdR17 supE44* Δ(*lac-proAB*) *endA1 gyrA96 relA1 thi* F′ (*traD36 proAB lacI*^{q} *lacZ*ΔM15)] was a gift of J. Andrews (University of Manchester, Manchester, United Kingdom). Strains JMtacA and JMtacB [JM109(pPH3) and JM109 (pAG111), respectively] were constructed in this laboratory (9). Strain EJ45 (*zeg298*::Tn*10 gyrA43*; temperature sensitive

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at 42°C) was a gift of G. Allison (University of Warwick, Warwick, United Kingdom). Strain HB2154 [Δ(*lac-proAB*) *ara14 thi-1* F′ (*proAB lacI*^{q} *lacZ*ΔM15) *mutL*::Tn*10*] and strain TG2 [Δ(*lac-proAB*) *supE thi hsdD5 recA*] were from D. B. Wigley (University of Leicester, Leicester, United Kingdom). Strain KA1106 [*E. coli* C600 *recA*(pRH43-117)] was from K. Abremski (du Pont). The construction of plasmid pPH3 was described previously (9). Plasmid pBR322 was prepared from strain JM109(pBR322), and plasmid pRH43-117 was prepared from strain KA1106. Plasmids were isolated by the alkaline lysis method of Birnboim and Doly (3).

Mutagenesis. Gap misrepair mutagenesis of plasmid pPH3 was carried out essentially as described by Hallett et al. (11). A 20-μg sample of supercoiled plasmid was singly nicked by incubation with DNase I (8×10^{-3} U; Sigma) in 50 mM Tris hydrochloride (pH 7.2)–5 mM $MgCl_2$–0.01% gelatin–150 μg of ethidium bromide per ml in a total volume of 200 μl in the dark for 1 h at 20°C. After extraction with water-saturated butan-1-ol, the DNA was precipitated with ethanol and resuspended in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. The nicked DNA was incubated with 20 U of exonuclease III (Boehringer) in 66 mM Tris hydrochloride (pH 7.5)–125 mM NaCl–0.66 mM $MgCl_2$–1 mM β-mercaptoethanol in a total volume of 200 μl for 5 min at 37°C. Control experiments showed that this treatment generated gaps of ~50 bp; gaps which were significantly larger were inefficiently repaired. The DNA was extracted with phenol and precipitated with ethanol. Misrepair of the nicked, gapped DNA was directed by incubating samples (0.4 μg) with 2 U of avian myeloblastosis virus reverse transcriptase (Anglian Biotec) and three of the four deoxynucleoside triphosphates (dNTPs) (5 mM each) in 50 mM Tris hydrochloride (pH 8.0)–100 mM KCl–50 mM $MgCl_2$–100 mM dithiothreitol in a total volume of 12 μl for 90 min at 37°C. The fourth dNTP (5 mM) was added, and incubation was continued for a further 90 min. After the addition of EDTA to a final concentration of 100 mM, the four reaction mixtures (each lacking one dNTP in the first avian myeloblastosis virus reverse transcriptase incubation) were pooled and the DNA was precipitated with ethanol and resuspended in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. Aliquots (0.2 μg) of the misrepaired plasmid were transformed into strain EJ45 made competent by the rubidium-MOPS method (12). Transformed cells were selected by plating onto Luria-Bertani agar plates containing ampicillin (50 μg/ml) and thymine (20 μg/ml) and incubating the plates at 30°C. Colonies from these plates were picked into duplicate 96-well microtiter plates, one of each pair containing 100 μl of LB broth plus ampicillin (50 μg/ml) and thymine (20 μg/ml) and the other containing the same but with the addition of CFX (0.5 μg/ml). All plates were incubated at 30°C in a shaking incubator for 2 h. Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.05 mM) was added to the wells of the CFX-containing plates, which were then transferred to a shaking incubator at 42°C. The plates with no CFX remained at 30°C. Growth in the CFX-containing plates may indicate that a quinolone resistance *gyrA* gene is present on the gap-misrepaired pPH3 plasmid. After growth was observed in a well on the CFX-containing plates, a sample was withdrawn from the corresponding well of the duplicate (non-CFX-containing) plate and the plasmid DNA by isolated with a microscale alkaline lysis procedure (3). After transformation into competent JM109 cells and selection on LB agar plates containing ampicillin, the strain was assessed for the ability to overproduce GyrA protein in liquid cultures following the addition of IPTG as described previously (9).

Site-directed mutagenesis was carried out essentially as described by Zoller and Smith (47). A 1.15-kbp *SstI-PstI* fragment (containing part of the *gyrA* gene from nucleotides 54 to 1208) derived from plasmid pPH3 was cloned into M13mp18. The mismatch oligonucleotide 5′-CATAGAC CGCGCGTCACCATGG-3′ (20 pmol) was annealed to the single-stranded form of the recombinant M13 (2 μg) and incubated with 2.4 U of the Klenow fragment of DNA polymerase I (Pharmacia) and 5 U of T4 DNA ligase (GIBCO/BRL) in 10 mM Tris hydrochloride (pH 8.1)–10 mM $MgCl_2$–5 mM dithiothreitol containing 0.25 mM each dATP, dGTP, dTTP, and dCTP at 4°C overnight. The DNA was transformed into *E. coli* HB2154 and added to an overnight culture of *E. coli* TG2 that had been mixed with soft agar and overlaid onto minimal agar plates. Plaques were picked onto plates, and colony screening was carried out by hybridization of the 5′-end-labeled mutagenic oligonucleotide. Putative positive colonies were analyzed by DNA sequencing by the dideoxy method (33) with Sequenase version 2.0 (United States Biochemicals) in accordance with the manufacturer's instructions.

Other methods. DNA gyrase A and B subunits were purified to homogeneity as described previously (9); specific activities of wild-type and mutant proteins were comparable to those previously reported. Cell extracts were made by resuspension of pelleted cells in 50 mM Tris hydrochloride (pH 7.5)–100 mM KCl–10% (wt/vol) glycerol–5 mM dithiothreitol–1 mM EDTA, disruption by sonication, and centrifugation. DNA supercoiling, relaxation, decatenation, and cleavage assays were carried out as described by Reece and Maxwell (31), except that the quinolone drugs NAL (Sigma), oxolinic acid (OXO; Sigma), NFX (gift of Merck Sharp and Dohme), and CFX (gift of Bayer) were added at the concentrations indicated. Determination of 50% inhibition of gyrase reactions by drugs was made by visual estimation of the amount of product as compared with that in a no-drug control. Protein concentrations were determined by the method of Bradford (4), and SDS-polycrylamide gel electrophoresis was carried out as described by Laemmli (19). Determination of 50% inhibitory concentrations was performed by a broth dilution method (5); 2×10^4 cells were inoculated into 100-μl aliquots of broth in microtiter plate wells at a range of drug concentrations and incubated overnight at 37°C (30°C in the case of strain EJ45). The 50% inhibitory concentration was defined as the minimum concentration of drug required to inhibit bacterial growth by 50%.

RESULTS

Mutagenesis. Gap misrepair mutagenesis is a technique for generating random point mutations in plasmid DNA in vitro (37). Plasmid pPH3, which contains the *gyrA* gene under the control of the *tac* promoter (9), was subjected to mutagenesis by this method. Of 188 putative drug-resistant clones screened, only 1 was found to be resistant to CFX at the concentration used (0.5 μg/ml). Preparation of the plasmid DNA from this clone and transformation into strain JM109 generated strain GM100. A cell extract was prepared from GM100 following induction of episomal *gyrA* expression by the addition of IPTG. Analysis of the cell extract by SDS-polyacrylamide gel electrophoresis confirmed the presence of a 97-kDa protein in large amounts. Supercoiling assays of the cell extract in the presence of CFX confirmed that a

TABLE 1. Fifty percent inhibitory concentrations for quinolone-susceptible and quinolone-resistant strains

Strain	Mutation	Temp (°C)	IPTG (0.05 mM)	50% Inhibitory concn of CFX (μg/ml)
EJ45		30	−	0.003
EJ45(pPH3)		30	−	0.003
EJ45(pPH3)		42	+	0.003
EJ45(pPH311.1)	Ser-83 (TCG)→Ala (GCG)	30	−	0.003
EJ45(pPH311.1)	Ser-83 (TCG)→Ala (GCG)	42	+	0.055
EJ45(pPH3100)	Gln-106 (CAG)→Arg (CGG)	30	−	0.003
EJ45(pPH3100)	Gln-106 (CAG)→Arg (CGG)	42	+	0.050

TABLE 2. Inhibition of wild-type and quinolone-resistant DNA gyrases by quinolone drugs

Assay	Quinolone	Drug concn (μg/ml) for:		
		JMtacA	SD11.1	GM100
50% Inhibition of supercoiling	CFX	0.05	0.5	0.5
	NFX	0.15	1.5	1.5
	OXO	1.5	5.0	5.0
	NAL	20.0	400.0	50.0
50% Inhibition of decatenation	CFX	0.1	1.0	ND[a]
50% Inhibition of relaxation	CFX	0.075	1.0	ND
50% Cleavage	CFX	0.1	1.0	1.0

[a] ND, Not determined.

quinolone-resistant GyrA protein was present (data not shown).

To determine the site of mutation, we exchanged restriction fragments from the mutant gene with those from the wild-type *gyrA* gene to generate chimeric genes in the manner described by Cullen et al. (5). This procedure revealed that the CFX resistance phenotype was conferred by a 0.6-kbp *Sst*I-*Sma*I fragment corresponding to nucleotide positions 54 to 636 in the *gyrA* sequence (39). Cloning of this fragment into M13mp19 and determination of the DNA sequence revealed the site of mutation to be an A→G transition at nucleotide 317 of the *gyrA* gene, resulting in the conversion of Gln-106 to Arg. The derivative of plasmid pPH3 bearing this mutation was named pPH3100.

The majority of the previously reported quinolone resistance mutations of the *gyrA* gene have resulted in the conversion of Ser-83 to either Leu or Trp (5, 45, 46). Indeed, it has been suggested that only these two substitutions at amino acid position 83 will lead to quinolone resistance (45). Both of these changes involve the conversion of a small polar amino acid (Ser) to bulky hydrophobic residues (Leu, Trp). To test whether the presence of a large hydrophobic group at this position is a prerequisite for quinolone resistance, we have effected the change Ser-83→Ala by using site-directed mutagenesis. Such a change involves virtually no alteration in the volume of the side chain while removing the polar hydroxyl group. By using site-directed mutagenesis, we have changed nucleotide 247 of the *gyrA* gene from T to G, resulting in the mutation Ser-83→Ala. An *Sst*I-*Xma*I restriction fragment carrying this change was exchanged with the corresponding fragment in pPH3 to generate plasmid pPH311.1. This plasmid was transformed in strain JM109, and the resultant strain was named SD11.1.

Plasmids pPH311.1 and pPH3100 were also transformed into the *gyrA*(Ts) strain EJ45. It has previously been shown that the presence of two *gyrA* alleles, one NAL susceptible and one NAL resistant, in the same strain leads to a quinolone susceptibility phenotype (13); i.e., susceptibility is dominant over resistance. So that our episomal *gyrA* genes could be expressed phenotypically, we used a *gyrA*(Ts) background and determined the 50% inhibitory concentrations at 30 and 42°C in the presence of the inducer IPTG (Table 1). We found that at 30°C both strains [EJ45 (pPH3100) and EJ45(pPH311.1)] showed wild-type levels of quinolone susceptibility. However, at 42°C in the presence of IPTG (Table 1), both strains were quinolone resistant, with 50% inhibitory concentrations about 18 times those of the wild type (Table 1). These results showed that both mutations, Gln-106→Arg and Ser-83→Ala, conferred quinolone resistance. At 42°C in the absence of IPTG, both mutant strains and EJ45(pPH3) showed no growth, implying that episomal *gyrA* expression occurs only in the presence of an inducer. This result confirms that expression of the *gyrA* gene is tightly controlled by the *tac* promoter in pPH3 (9).

Enzymatic properties of mutant proteins. The DNA gyrase A proteins from the quinolone-resistant mutants GM100 and SD11.1 were purified (9), and their enzymatic activities were compared with that of the wild-type DNA gyrase A protein purified from JMtacA. Assay of the DNA supercoiling activities of the mutant A proteins in the presence of the B proteins showed these A proteins to be very similar to the wild-type A protein (data not shown). This result indicates that the mutations Ser-83→Ala and Gln-106→Arg do not significantly alter the supercoiling activity of the enzyme and hence probably do not affect a rate-limiting step in this process. The concentrations of various quinolone drugs required to inhibit supercoiling by 50% were determined (Table 2). For the fluoroquinolones (CFX and NFX), the 50% inhibitory concentrations for the mutant gyrases were 10 times that for the wild-type enzyme. Inhibition by OXO required about three times more drug for the mutants than for the wild type. Inhibition of supercoiling by NAL differed for the two mutants. Gyrase containing the mutation Ser-83→Ala required 20 times more drug for the inhibition of supercoiling than did the wild type, whereas gyrase containing the mutation Gln-106→Arg required only 2.5 times more drug (Table 2). Although these assays are not strictly quantitative, the results indicate that different quinolones may interact with amino acids in GyrA in different ways.

Although DNA supercoiling is the most frequently used assay for DNA gyrase, it is not the only measurable activity of the enzyme, nor does it necessarily reflect the role of the enzyme in vivo. For example, it has been proposed that gyrase may be involved in the relaxation and decatenation of DNA in the cell (22, 38). Therefore, we have assessed the sensitivity of several reactions of wild-type and quinolone-resistant DNA gyrases to inhibition by CFX. One aim of these experiments was to establish whether the sensitivity of these other gyrase reactions to quinolones more closely parallels the 50% inhibitory concentrations.

In the absence of ATP, DNA gyrase relaxes negatively supercoiled DNA (8). We have found that DNA relaxation by wild-type and quinolone-resistant gyrases is inhibited at CFX concentrations similar to those that inhibit the corresponding supercoiling reactions (Table 2). It is thought that the decatenation of DNA circles may reflect an essential

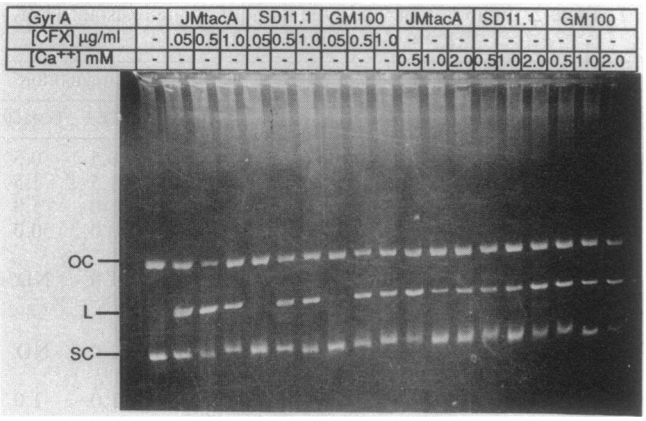

FIG. 1. Induction of DNA cleavage by wild-type and quinolone-resistant DNA gyrases in the presence of CFX and Ca^{2+}. Wild-type GyrB protein was complexed with either wild-type (JMtacA) or quinolone-resistant (SD11.1: Ser-83→Ala; GM100: Gln-106→Arg) GyrA protein, as indicated, at a final gyrase (A_2B_2) concentration of 40 nM and incubated with supercoiled pBR322 DNA (10 μg/ml) in the presence of CFX or $CaCl_2$ at the indicated concentrations for 1 h at 25°C. Reactions were terminated by the addition of SDS (0.2%) and proteinase K (0.1 mg/ml), and samples were incubated for a further 30 min at 37°C. Samples were subjected to electrophoresis on a 1% agarose gel. OC, Nicked circular DNA; L, linear; SC, supercoiled.

function of gyrase in the cell, namely, the separation of daughter chromosomes at the termination stage of replication (38). We have generated catenated DNA circles by using the reaction of cre recombinase on plasmid pRH43-117, which contains two *loxP* sites, one wild type and one mutant. DNA gyrase can resolve these catenanes into free-product circles (31). We have examined this decatenation reaction by using wild-type and quinolone-resistant gyrases. It has previously been shown that decatenation by gyrase is an ATP-dependent reaction (24). We have confirmed this result and shown that the reaction also does not occur in the presence of the nonhydrolyzable ATP analog 5′-adenylyl-β,γ-imidodiphosphate (10). We do not presently understand the energy requirement of the decatenation reaction. We have found that the amounts of CFX required to inhibit the decatenation reactions of the wild-type and mutant enzymes correspond closely to those required to inhibit supercoiling (Table 2).

In the presence of quinolone drugs, the denaturation of the gyrase-DNA complex by SDS results in a double-strand DNA break (8). This reaction may be used to assess the potency of different quinolone drugs (2). Figure 1 shows the quinolone-induced cleavage of DNA by wild-type and quinolone-resistant gyrases. DNA cleavage by the mutant gyrases requires about 10-fold more drug than does that by the wild-type enzyme. This difference is consistent with the CFX 50% inhibitory concentrations for supercoiling with wild-type and mutant enzymes. DNA cleavage by gyrase has also been observed when Ca^{2+} is substituted for Mg^{2+} in the reaction (31). Figure 1 shows that the Ca^{2+}-induced cleavage of DNA by gyrase is similar for wild-type and quinolone-resistant gyrases at the $CaCl_2$ concentrations used (0.5 to 2 mM). The lowest $CaCl_2$ concentration at which cleavage is detectable under these conditions is 0.1 mM for both wild-type and mutant enzymes (data not shown).

DISCUSSION

One approach to the study of the mode of action of quinolone drugs on DNA gyrase is to isolate and characterize quinolone-resistant gyrase proteins. By determining the amino acid the alteration of which leads to the resistance phenotype, some insight into the nature of the drug-enzyme interaction may be gained. Moreover, study of the enzymatic reactions of the mutant proteins in vitro may shed light on the mode of drug action. Several quinolone-resistant mutants of DNA gyrase have been described. These represent seven mutations of the *gyrA* gene (Ala-67→Ser, Gly-81→Cys, Ser-83→Leu, Ser-83→Trp, Ala-84→Pro, Asp-87→Asn, and Gln-106→His) (5, 45, 46) and two mutations of the *gyrB* gene (Asp-426→Asn and Lys-447→Glu) (43). Such mutations in the *gyrA* gene lead to increases in MICs of between 4- and 30-fold. The mutations identified so far have arisen spontaneously.

For this study, we have generated two novel quinolone-resistant mutants by using site-directed and gap misrepair mutageneses. Both of these methods are generally applicable to the mutagenesis of the *gyrA* gene and could be utilized to generate mutations at many other loci in the gene. (A previous attempt to apply gap misrepair mutagenesis to plasmid pMK90, which contains the *gyrA* gene under the partial control of the λ p_L promoter [25], resulted in the identification of a 60-kDa protein unrelated to GyrA but highly homologous to the heat shock protein GroEL [11].) The availability of plasmid pPH3, which encodes the *gyrA* gene under the tight control of the *tac* promoter (9), greatly facilitates these mutagenesis methods and permits the production of large amounts of the mutant proteins. One possible drawback of the gap misrepair strategy that we used was that potential mutants had to be screened in liquid cultures. We have found that the *gyrA*(Ts) strain EJ45 does not grow on plates at 42°C in the presence of CFX, even when harboring an episomal quinolone resistance *gyrA* gene, e.g., EJ45(pPH311.1). We do not presently understand the reasons for this phenomenon. We were unable to use the alternative *gyrA*(Ts) strain KNK453 (17) in this strategy, as it possesses a *polA* mutation and will not support the replication of plasmid pPH3.

Most of the quinolone resistance mutations isolated in the *gyrA* gene so far are at amino acid Ser-83 (5, 45, 46). Despite there being several possible amino acid substitutions at this position which could be generated by point mutations, only changes to Leu and Trp have been reported so far (5, 45, 46). Indeed, it has been speculated that only such bulky hydrophobic residues will confer the quinolone resistance phenotype when present at position 83 (45). We have shown that the more conservative change of Ser-83→Ala also results in a quinolone-resistant GyrA protein. This result suggests that the loss of the hydroxyl group alone is responsible for the resistance phenotype. That the 50% inhibitory concentration of CFX for strains containing this protein (Table 1) is lower than are those for strains with the Ser-83→Leu and Ser-83→Trp mutations (5, 45, 46) implies that the bulkiness of the Leu and Trp residues contributes further to the level of quinolone resistance, presumably by further destabilizing the gyrase-quinolone interaction. With regard to Gln-106, a mutation to histidine has been the only mutation so far reported to lead to quinolone resistance at this position (45, 46). The CFX MIC for the strain bearing this mutation is low, being only four times that for the wild type. It has previously been suggested that Gln-106 represents a site at which mutation leads to low-level resistance (45). By con-

trast, we have found that the mutation Gln-106→Arg induces a level of resistance comparable to that induced by the mutation Ser-83→Ala, suggesting that the level of resistance depends on the specific amino acid substituted. It is interesting to note that both mutations identified at Gln-106 are to positively charged residues (His, Arg), suggesting that a positive charge substituted for a polar group at this position may destabilize the gyrase-quinolone interaction.

It has previously been pointed out that the quinolone resistance mutations so far mapped in the *gyrA* gene cover only a small area of the protein clustered near Tyr-122, which is the site of covalent attachment of the DNA; this region has been termed the quinolone resistance-determining region (45). This region of the protein (between amino acids 67 and 106) may form the site of interaction with the quinolone drugs. That three of these amino acids are hydrophilic (Ser-83, Asp-87, and Gln-106) may support the idea that they are involved in hydrogen-bonding interactions with the drug molecules. Such a suggestion is inconsistent with the current model of the quinolone-gyrase interaction, in which hydrogen-bonding interactions are proposed to occur between the drug and hydrogen bond donors on the DNA and enzyme-drug interactions are proposed to occur between the group at position 7 of the quinolone and DNA gyrase (35).

One of the goals of the present study was to investigate whether there is a correlation between the MICs of quinolones and the ability of the drugs to inhibit various reactions of DNA gyrase. The results in Table 2 indicate that for all the reactions tested, approximately 10 to 30 times more drug is needed to inhibit the in vitro reactions than to exert the bactericidal effect in vivo for both wild-type and mutant enzymes. Although the quantitation of such reactions is imprecise, these results tend to argue against the primary mode of action of quinolones being the inhibition of these gyrase activities in vivo. In all cases, increased CFX concentrations are required to inhibit the in vitro reactions of the mutant enzymes. These increased concentrations mirror the increased 50% inhibitory concentrations for strains harboring the mutant versus the wild-type enzyme (Table 1). One interesting observation is that DNA cleavage induced by Ca^{2+} is very similar for wild-type and mutant enzymes (Fig. 1). This result suggests that the mutations Ser-83→Ala and Gln-106→Arg do not affect the cleavage reaction per se but destabilize the interaction of the enzyme with the quinolone drugs. It is not known how Ca^{2+} interrupts the breakage-reunion reaction of gyrase.

One hypothesis which explains the effects of quinolone drugs on DNA gyrase in vivo and accounts for the MIC paradox is that the binding of the drug to the enzyme converts it into a "poison." This hypothesis, first suggested by Kreuzer and Cozzarelli (17), proposes that the drug-enzyme-DNA complex represents a trapped reaction intermediate which can inhibit DNA replication and other processes. Drlica et al. (6) further proposed that the poison was likely to act close to DNA replication forks and suggested that gyrase may have a role in the facilitation of replication fork movement. The suggestion that quinolone drugs act as poisons in the cell corresponds to the suggested action of eucaryotic topoisomerase II inhibitors, which have been proposed to act in the same way (21, 27).

The poison hypothesis explains the observed dominance of quinolone-susceptible over quinolone-resistant *gyrA* alleles in partial diploids (13). Thus, a cell containing quinolone-susceptible GyrA subunits, even in small amounts, will always be susceptible, irrespective of the presence of quinolone-resistant GyrA. Indeed, we have found that bacteria containing an episomal quinolone resistance *gyrA* gene will only be phenotypically resistant if the chromosomal quinolone susceptibility *gyrA* gene can be inactivated, as in the *gyrA*(Ts) strain EJ45 at 42°C (Table 1). Therefore, this hypothesis suggests that MICs would correlate with quinolone drug concentrations at which relatively few gyrase molecules have bound the drug. The current in vitro assays (Table 2) are not sensitive to such low levels of gyrase-drug complex. Walton and Elwell (40) have used a modified gyrase cleavage assay in which radiolabeled DNA is used as the substrate, resulting in the detection of lower levels of cleavage than in the standard assay (e.g., Fig. 1) and a better correlation with MICs (within a factor of two in some cases).

With the identification of a number of quinolone resistance loci in *gyrA* (5, 45, 46) and the availability of the mutant proteins in large amounts, it should now be possible to address further questions concerning gyrase-quinolone interactions. For example, other amino acid substitutions could be made at the known quinolone resistance loci to further probe the nature of the protein-drug interaction. Other experiments could test quinolone binding with wild-type and resistant proteins, study the effects of quinolones on in vitro DNA replication involving wild-type and resistant gyrases, and study the structures of quinolone-susceptible and -resistant gyrase proteins. Recently, a 64-kDa tryptic fragment of the GyrA protein has been isolated and shown to be able to perform the quinolone-induced DNA cleavage reaction in the presence of the GyrB protein (31). This fragment can now be made as a gene product (32) and has been crystallized and shown to diffract X rays (30). It is hoped that structural work on the wild-type protein and similar work on quinolone-resistant proteins will facilitate the design and development of other more potent quinolone drugs.

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REFERENCES

1. **Adachi, T., M. Mizuuchi, E. A. Robinson, E. Apella, M. H. O'Dea, M. Gellert, and K. Mizuuchi.** 1987. DNA sequence of the *E. coli gyrB* gene: application of a new sequencing strategy. Nucleic Acids Res. **15**:771–784.
2. **Barrett, J. F., J. A. Sutcliffe, and T. D. Gootz.** 1990. In vitro assays used to measure the activity of topoisomerases. Antimicrob. Agents Chemother. **34**:1–7.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Cullen, M. E., A. W. Wyke, R. Kuroda, and L. M. Fisher.** 1989. Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers resistance to 4-quinolones. Antimicrob. Agents Chemother. **33**:886–894.
6. **Drlica, K., E. C. Engle, and S. H. Manes.** 1980. DNA gyrase on the bacterial chromosome: possibility of two levels of action. Proc. Natl. Acad. Sci. USA **77**:6879–6883.
7. **Gellert, M.** 1981. DNA topoisomerases. Annu. Rev. Biochem. **50**:879–910.
8. **Gellert, M., K. Mizuuchi, M. H. O'Dea, H. Ohmori, and J.-I.**

Tomizawa. 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74**:4772–4776.

9. **Hallett, P., A. J. Grimshaw, D. B. Wigley, and A. Maxwell.** 1990. Cloning of the DNA gyrase genes under *tac* promoter control: over-production of the gyrase A and B proteins. Gene **93**:139–142.
10. **Hallett, P., A. Maxwell, and R. J. Reece.** Unpublished data.
11. **Hallett, P., A. Mehlert, and A. Maxwell.** 1990. *Escherichia coli* cells resistant to the DNA gyrase inhibitor, ciprofloxacin, overproduce a 60 kD protein homologous to GroEL. Mol. Microbiol. **4**:345–353.
12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **129**:375–392.
13. **Hane, M. W., and T. H. Wood.** 1969. *Escherichia coli* K-12 mutants resistant to nalidixic acid: genetic mapping and dominance studies. J. Bacteriol. **99**:238–241.
14. **Horowitz, D. S., and J. C. Wang.** 1987. Mapping the active site tyrosine of *Escherichia coli* DNA gyrase. J. Biol. Chem. **262**: 5339–5344.
15. **Hussain, K., E. J. Elliot, and G. P. C. Salmond.** 1987. The ParD$^-$ mutant of *Escherichia coli* also carries a *gyrA*am mutation. The complete sequence of *gyrA*. Mol. Microbiol. **1**:259–273.
16. **Klevan, L., and J. C. Wang.** 1980. Deoxyribonucleic acid gyrase-deoxyribonucleic acid complex containing 140 base pairs of deoxyribonucleic acid and an $\alpha_2\beta_2$ core. Biochemistry **19**: 5229–5234.
17. **Kreuzer, K. N., and N. R. Cozzarelli.** 1979. *Escherichia coli* mutants thermosensitive for deoxyribonucleic acid gyrase subunit A: effects on deoxyribonucleic acid replication, transcription, and bacteriophage growth. J. Bacteriol. **140**:424–435.
18. **Krueger, S., G. Zaccai, A. Wlodawer, J. Langowski, M. O'Dea, A. Maxwell, and M. Gellert.** 1990. Neutron and light-scattering studies of DNA gyrase and its complex with DNA. J. Mol. Biol. **211**:211–220.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lesher, G. Y., E. J. Froelich, M. D. Gruett, J. H. Bailey, and R. P. Brundage.** 1962. 1,8-Naphthyridine derivatives. A new class of chemotherapeutic agents. J. Med. Chem. **5**:1063–1065.
21. **Liu, L. F.** 1989. DNA topoisomerase poisons as antitumor drugs. Annu. Rev. Biochem. **58**:351–375.
22. **Liu, L. F., and J. C. Wang.** 1987. Supercoiling of the DNA template during transcription. Proc. Natl. Acad. Sci. USA **84**:7024–7027.
23. **Maxwell, A., and M. Gellert.** 1986. Mechanistic aspects of DNA topoisomerases. Adv. Protein Chem. **38**:69–107.
24. **Mizuuchi, K., L. M. Fisher, M. H. O'Dea, and M. Gellert.** 1980. DNA gyrase action involves the introduction of transient double-strand breaks into DNA. Proc. Natl. Acad. Sci. USA **77**:1847–1851.
25. **Mizuuchi, K., M. Mizuuchi, M. H. O'Dea, and M. Gellert.** 1984. Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. J. Biol. Chem. **259**:9199–9201.
26. **Nakamura, S., M. Nakamura, T. Kojima, and H. Yoshida.** 1989. *gyrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*. Antimicrob. Agents Chemother. **33**:254–255.
27. **Nelson, E. M., K. M. Tewey, and L. F. Liu.** 1984. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-aniside. Proc. Natl. Acad. Sci. USA **81**:1361–1365.
28. **Neu, H. C.** 1989. Clinical utility of DNA gyrase inhibitors. Pharmacol. Ther. **41**:207–221.
29. **Piddock, L. J. V.** 1989. Resistance to quinolones and fluoroquinolones, p. 169–192. *In* L. E. Bryan (ed.), Handbook of experimental pharmacology, vol. 91. Springer-Verlag KG, Berlin.
30. **Reece, R. J., Z. Dauter, K. S. Wilson, A. Maxwell, and D. B. Wigley.** 1990. Preliminary crystallographic analysis of the 64kDa breakage-reunion domain of *Escherichia coli* DNA gyrase A protein. J. Mol. Biol. **215**:493–495.
31. **Reece, R. J., and A. Maxwell.** 1989. Tryptic fragments of the *Escherichia coli* DNA gyrase A protein. J. Biol. Chem. **264**: 19648–19653.
32. **Reece, R. J., and A. Maxwell.** J. Biol. Chem., in press.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
34. **Shen, L. L., W. C. Kohlbrenner, D. Weigl, and J. Baranowski.** 1989. Mechanism of quinolone inhibition of DNA gyrase. J. Biol. Chem. **264**:2973–2978.
35. **Shen, L. L., L. A. Mitscher, P. N. Sharma, T. J. O'Donnell, D. T. W. Chu, C. S. Cooper, T. Rosen, and A. G. Pernet.** 1989. Mechanisms of inhibition of DNA gyrase by quinolone antibacterials: a cooperative drug-DNA binding model. Biochemistry **28**:3886–3894.
36. **Shen, L. L., and A. G. Pernet.** 1985. Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. Proc. Natl. Acad. Sci. USA **82**:307–311.
37. **Shortle, D., P. Grisafi, S. Benkovic, and D. Botstein.** 1982. Gap-misrepair mutagenesis: efficient site-directed induction of transition, transversion and frameshift mutations *in vitro*. Proc. Natl. Acad. Sci. USA **79**:1588–1592.
38. **Steck, T. R., and K. Drlica.** 1984. Bacterial chromosome segregation: evidence for DNA gyrase involvement in decatenation. Cell **36**:1081–1088.
39. **Swanberg, S. L., and J. C. Wang.** 1987. Cloning and sequencing of the *Escherichia coli gyrA* gene coding for the A subunit of DNA gyrase. J. Mol. Biol. **197**:729–736.
40. **Walton, L., and L. P. Elwell.** 1988. In vitro cleavable-complex assay to monitor antimicrobial potency of quinolones. Antimicrob. Agents Chemother. **32**:1086–1089.
41. **Wang, J. C.** 1985. DNA topoisomerases. Annu. Rev. Biochem. **54**:665–697.
42. **Wolfson, J. S., and D. C. Hooper.** 1985. The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity in vitro. Antimicrob. Agents Chemother. **28**:581–586.
43. **Yamagishi, J., H. Yoshida, M. Yamayoshi, and S. Nakamura.** 1986. Nalidixic acid-resistant mutations of the *gyrB* gene of *Escherichia coli*. Mol. Gen. Genet. **204**:367–373.
44. **Yamagishi, J.-I., Y. Furutani, S. Inoue, T. Ohue, S. Nakamura, and M. Shimizu.** 1981. New nalidixic acid resistance mutations related to deoxyribonucleic acid gyrase activity. J. Bacteriol. **148**:450–458.
45. **Yoshida, H., M. Bogaki, M. Nakamura, and S. Nakamura.** 1990. Quinolone resistance-determining region of the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **34**:1271–1272.
46. **Yoshida, H., T. Kojima, J. Yamagishi, and S. Nakamura.** 1988. Quinolone-resistant mutations of the *gyrA* gene of *Escherichia coli*. Mol. Gen. Genet. **211**:1–7.
47. **Zoller, M., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100**:468–500.
48. **Zweerink, M. M., and A. Edison.** 1986. Inhibition of *Micrococcus luteus* DNA gyrase by norfloxacin and 10 other quinolone carboxylic acids. Antimicrob. Agents Chemother. **29**:598–601.