Cloning and Characterization of a DNA Gyrase A Gene from *Escherichia coli* That Confers Clinical Resistance to 4-Quinolones

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Nalidixic acid, enoxacin, and other antibacterial 4-quinolones inhibit DNA gyrase activity by interrupting DNA breakage and reunion by A subunits of the A_2B_2 gyrase complex. Despite their clinical importance, the mode of quinolone action and mechanisms of resistance are poorly understood at the molecular level. Using a DNA fragment enrichment procedure, we isolated the *gyrA* gene from a uropathogenic *Escherichia coli* strain that encodes a gyrase A protein cross-resistant to a variety of quinolones. When complemented with gyrase B subunit, the purified A protein reconstituted DNA supercoiling activity ~100-fold more resistant to inhibition by enoxacin than the susceptible enzyme and failed to mediate quinolone-dependent DNA cleavage. Nucleotide sequence analysis revealed that the gene differed at 58 nucleotide positions compared with the K-12 *gyrA* sequence. The 875-amino-acid residue-resistant gyrase A protein differed at three positions from its wild-type *E. coli* K-12 counterpart: tryptophan, glutamate, and serine replaced serine, aspartate, and alanine residues at positions 83, 678, and 828, respectively. By genetic analysis of chimeric *gyrA* genes in a *gyrA* locus background, we showed that the Ser-83→Trp mutation in the gyrase A protein was solely responsible for high-level bacterial resistance to nalidixic acid and fluoroquinolones.

Bacterial DNA is maintained in a negatively supercoiled state by DNA gyrase, an ATP-dependent type II DNA topoisomerase (10). Gyrase is essential for cell viability, being implicated in a range of DNA transactions, including DNA replication and recombination, and in the control of gene expression (8, 35). Gyrase catalyzes DNA supercoiling by an interesting and unusual mechanism. It passes a duplex DNA segment through a transient double-stranded DNA break made within a 120- to 150-base-pair (bp) loop of DNA wrapped on the surface of the tetrameric A_2B_2 gyrase complex. DNA strand passage is the salient mechanistic feature that allows DNA supercoiling and ATP-independent DNA relaxation by gyrase and also accounts for its catenation or decatenation and DNA unknotting activities (3, 16, 18).

Antibacterial quinolones such as oxolinic acid inhibit DNA supercoiling by gyrase in vitro and rapidly arrest DNA replication in vivo (2, 9, 12, 28). Addition of detergent to gyrase-DNA complexes formed in the presence of oxolinic acid results in site-specific double-stranded DNA breakage and covalent attachment of the gyrase A subunits to each 5'-phosphate end via tyrosine (Tyr)-122 (6, 7, 9, 15, 22, 31). Thus, the A subunits appear to promote DNA breakage and reunion during catalysis, a process interrupted by quinolone inhibitors. In contrast, the B subunits bind ATP and are the locus of action of coumarin antibiotics such as novobiocin (11, 20, 30). The A and B subunits can be individually purified but must be combined to generate the topoisomerase activities of gyrase (14, 19).

The molecular basis underlying the enzymatic and potent antibacterial effects of quinolones is not yet understood. Given the current clinical and mechanistic interest in quinolones (36), we have sought to elucidate their mode of action by characterizing quinolone resistance in clinical isolates of *Escherichia coli*. Here we report the genetic and enzymatic characterization of the *gyrA* gene and its quinolone-resistant gyrase A product from a resistant uropathogenic *E. coli* strain.

**MATERIALS AND METHODS**

**Materials.** *E. coli* strains used in this study were the following: RW1053 recA Δ(bio att gal), MK47 and N4186 (19), DH5 recA gyrA96 (Nal') (from Bethesda Research Laboratories, Gaithersburg, Md.), and KNK453 gyrA43(Ts) (from Ken Kreuzer, University of North Carolina, Chapel Hill). Clinical isolate 227 was kindly donated by Laura Piddock, The University of Birmingham, Birmingham, United Kingdom, and was confirmed as *E. coli* by API tests (Analytab Products, Plainview, N.Y.). The strain was isolated in February 1985 from the urine of a patient at All Saints Hospital, Birmingham, United Kingdom. The patient, part of a geriatric study, received 200 mg of enoxacin twice daily for 5 days. Strain 227 carried a 6-kilobase (kb) plasmid conferring resistance to ampicillin at 25 μg/ml. Nalidixic acid (Sterling Winthrop), norfloxacin (Merck & Co., Inc., Rahway, N.J.); ofloxacin, enoxacin, and ciprofloxacin (Bayer); pefloxacin (Bellon); gentamicin (Roussel); and cefazidime (Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom) were obtained either from the appropriate company or through Glaxo. Chloramphenicol, novobiocin, and low-gelling agarose were from Sigma Chemical Co., St. Louis, Mo. [γ-32P]ATP and [α-32P]ATP (each 3,000 Ci/mmol), T4 polynucleotide kinase, restriction enzymes, Hybond-N, and Multiscribe DNA labeling kits were from Amersham International plc, Little Chalfont, United Kingdom. Avian myeloblastosis virus reverse transcriptase and the ampicillin resistance plasmid pSPT19, calf intestinal alkaline phosphatase and T4 DNA ligase, and protease K were from Pharmacia, Uppsala, Sweden; Boehringer GmbH, Mannheim, Federal Republic of Germany; and BDH Chemicals, Poole, United Kingdom, respectively. DNA polymerase I (Klenow fragment) and NcoI were from New England Bio-

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VOL. 33, LABS, INC., BEVERLY, MASS. LOW-GEELLING AGAROSE (TYPE VII) was obtained from Sigma Chemical Co. Novobiocin-Sepharose was prepared as described previously (29). Gyrase A and B proteins were purified to near homogeneity from overproducing strains N4186 and MK47 (19). High-molecular-weight chromosomal DNA from E. coli cultures was isolated as described previously (25). Plasmid pRM386 carrying the wild-type K-12 gyrA gene was kindly given by Rolf Menzel, E. I. du Pont Experimental Station, Wilmington, Del. Plasmid pBR322 was prepared as described previously (18). Plasmid pSPT19 was from Boehringer.

**Drug susceptibility of *E. coli* strains.** MICs of various antibiotics were determined by the standard broth dilution method, using microdilution plates and an inoculum of 2×10⁶ cells per well. Turbidity was scored either visually or by using a Titertek Multiskan microdilution plate reader.

**Southern blotting.** Genomic restriction maps for the gyrA gene in *E. coli* 227 or RW1053 were determined as follows. Chromosomal DNA was digested with either *Sacl* or *HindIII* and one of several other restriction enzymes. DNA fragments were separated on 0.8% agarose gels, transferred to nitrocellulose or Hybond-N, and probed with a nick-translated or randomly primed 600-bp *Sacl-HindIII* or a 2.8-kb *HindIII* fragment from within the wild-type gyrA gene. This indirect end-labeling approach (37) allowed sites to be located relative to the *Sacl* and *HindIII* sites internal to the gyrA gene.

**DNA cloning of the strain 227 gyrA gene.** Genomic DNA was digested with *BamHI* and *KpnI*, and the DNA fragments were separated on a 0.8% low-gelling agarose gel. DNA size markers were run in outside lanes, and these sections of the gel were excised, stained with ethidium bromide, and photographed. The unstained gel was cut into slices, and then DNA in the 7- to 8-kb range was purified from the gel by phenol extraction and ethanol precipitation. The DNA was ligated into *BamHI-KpnI*-cut plasmid pSPT19 and used to transform highly competent *E. coli* DH5 cells, which were then plated on L-ampicillin plates (25 μg/ml) overlaid with Hybond-N filters and incubated at 37°C overnight. Colonies were replica plated, and replica Hybond-N filters were incubated overnight on L-chloramphenicol plates (10 μg/ml) to amplify plasmid copy number. Cells were lysed and the DNA was fixed on the filters by UV irradiation. Duplicate filters were hybridized at high stringency to a 600-bp *Sacl-HindIII* fragment from the 5' end of the K-12 gyrA gene (Fig. 1) excised from plasmid pRM386 and labeled with [α-32P]dCTP by random priming. Strongly hybridizing colonies were picked from the master plate and grown on a large scale, and their plasmids were isolated and restriction mapped. Two independent positive clones were obtained which contained identical plasmids with 7.5-kb inserts. Both plasmids had functional gyrA genes as demonstrated by their ability to confer temperature-independent growth on strain KNK453, which harbors a gyrA(Ts) allele.

**DNA sequence analysis.** The 5'-end-labeling, using T4 polynucleotide kinase, was carried out as described previously (6). The 3'-end-labeling of *HindIII* sites was done in 100 mM Tris hydrochloride, pH 8.0–10.0 mM MgCl₂–6 mM 2-mercaptoethanol (15 μl), using [α-32P]dATP (15 μCi) and reverse transcriptase (1 U). After 2 h at 37°C, an equal volume of 8 M ammonium acetate was added and the DNA was ethanol precipitated. For *BglII* ends, GTP (50 μM) was also included. DNA fragments labeled at a unique 3' or 5' end were obtained by recutting with a second restriction enzyme. Sequencing reactions were carried out by the chemical method of Maxam and Gilbert (17).

**FIG. 1.** Restriction maps for the gyrA locus in *E. coli* 227 (a) and RW1053 (b). The gyrA gene is indicated by the heavy line. B, C, H, K, and S denote restriction sites for *BamHI*, *ClaI*, *HindIII*, *KpnI*, and *Sacl*, respectively. The gyrA gene was localized and restriction maps were determined by Southern blotting of appropriately digested chromosomal DNA, using 600-bp *Sacl-HindIII* and 2.8-kb *HindIII* probes derived from the *E. coli* K-12 gyrA gene (32) (shown by brackets) and the indirect end-labeling method (37).

The sequencing strategy initially involved labeling at restriction sites in pMEC5 for enzymes with six base recognition sequences, viz., *BglII*, *HindIII*, *ClaI*, *PvuI*, *NcoI*, and *Sacl*. After secondary cutting, singly end-labeled restriction fragments from within the gyrA gene were isolated and sequenced. Subsequently, specific gyrA restriction fragments were purified on low-gelling agarose gels, namely, a 3.9-kb *Sacl-NruI* fragment, a 900-bp *BglII* fragment, a 600-bp *Sacl-HindIII* fragment, and a 2.0-kb *BamHI-Sacl* fragment containing the 5' region of the gene. When appropriate, these fragments were 5' labeled with *HinfI*, *TaqI*, *MspI*, *Rsal*, or *HaeIII* sites. Radiolabeled fragments were separated on 2% low-gelling agarose gels and purified by phenol extraction and ethanol precipitation. After digestion with a second restriction enzyme, end-labeled fragments were again purified on low-gelling agarose.

Denaturing polyacrylamide (10%) sequencing gels were run as described by Fisher et al. (6). Gels were wrapped in cling film and autoradiographed at −70°C by exposure to preflashed Fuji X-ray film, using a light-intensifying screen. Nucleotide changes were verified by sequencing both DNA strands at least twice.

**Chimeric gyrA genes.** Plasmids pRM386 and pMEC5 were each digested with *BamHI* and *SmaI*, and each pair of fragments was separated and purified from low-gelling agarose. Heterologous pairs of *BamHI-SmaI* DNA fragments were ligated and used to transform *KnK453*, RW1053, or DH5. Ampicillin-resistant colonies were restreaked, and plasmids were then isolated by the rapid alkaline extraction method prior to restriction mapping. Plasmids pMEC6 and pMEC7 had the expected restriction maps. The quinolone susceptibility of bacteria transformed with these plasmids was examined by the broth dilution method. For DH5 cells, susceptibility to nalidixic acid was examined on L-broth plates containing 50 μg of drug per ml at 37°C.

**Purification of gyrase A protein and DNA breakage assays.** For use in DNA cleavage experiments, strain 227 gyrA protein was purified free of trace endonuclease contamination by affinity chromatography (29). Cells were grown in 10 liters of L broth to an optical density at 600 nm of ~1.5, pelleted, washed with 50 mM Tris hydrochloride, pH 8.0–10% glycerol–1 mM dithiothreitol, and stored at −70°C as described previously (19). Bacteria were thawed and treated with lysozyme, and the gyrase A protein was purified by Polymun P and ammonium sulfate precipitation. Protein from
the ammonium sulfate pellet was dissolved in 50 mM Tris hydrochloride, pH 7.5–10% glycerol–1 mM EDTA–5 mM dithiothreitol and loaded on a novobioncin-Sepharose column (500 μl). The column was washed with the same buffer, and gyrase A protein was eluted with 50 mM Tris hydrochloride, pH 7.5–10% glycerol–1 mM trisodium EDTA–5 mM dithiothreitol containing 1 M KCl. Peak fractions contained a subunit >90% pure by polyacrylamide gel analysis. Purified A subunit was unable to catalyze DNA supercoiling in the absence of added gyrase B protein.

DNA supercoiling activity was assayed with relaxed pBR322 DNA essentially as described by Gellert et al. (10), using excess purified gyrase B subunit. One unit of gyrase activity supercoils 50% of the input DNA at 37°C in 30 min.

Quinolone-mediated cleavage of supercoiled pBR322 DNA (0.6 μg) was examined, using gyrase A (25 U) and gyrase B (25 U) proteins in the absence or presence of enoxacin under the conditions described by Fisher et al. (6).

DNA samples from supercoiling or cleavage experiments were electrophoresed on 0.8% agarose gels by the method of Fisher et al. (6).

**RESULTS**

**Cloning the quinolone-resistant gyrA gene from a clinical isolate by DNA fragment enrichment.** We have described previously an *E. coli* isolate, strain 227, taken from a patient treated for a urinary tract infection with multiple doses of enoxacin over several days (M. E. Cullen, A. W. Wyke, F. McCaughen, C. A. Austin, and L. M. Fisher, Curr. Top. Infect. Dis. Clin. Microbiol., in press). Strain 227 is resistant to enoxacin and cross-resistant to other quinolones (Table 1). We have shown that gyrase A protein partially purified from the strain reconstitutes a quinolone-resistant gyrase activity when tested in a DNA supercoiling assay. The results establish that the strain carries one or more quinolone-resistant mutations in its gyrA gene encoding the gyrase A subunit.

Quinolone-susceptible gyrA alleles are usually dominant over resistant alleles (13), and thus it was not feasible to clone the 227 gyrA gene by simple drug selection. We therefore chose a colony hybridization method, using wild-type gyrA probes.

First, a genomic restriction map for the gyrA locus in strain 227 was determined by Southern blotting (Fig. 1a). Genomic 227 DNA was digested with both SacI and HindIII restriction enzymes and probed with a radioabeled 600-bp SacI-HindIII fragment from within the K-12 gyrA gene (shown in Fig. 1). The same 600-bp fragment was detected in the 227 genomic DNA, indicating the presence of conserved SacI and HindIII sites in the 227 gyrA gene. The 227 DNA was then doubly digested with SacI or HindIII and one of several other restriction enzymes and probed with the 600-bp SacI-HindIII fragment. The sizes of the resulting hybridizing fragments enabled restriction sites to be located 3′ of the SacI site (for double digests involving SacI) and 5′ of the HindIII site (for those digestes incorporating HindIII). As the size of the *E. coli* K-12 gyrA gene is known to be 2.6 kb (32), it could be deduced that the 227 gene lies on a 7.5-kb BamHI-KpnI fragment. The 227 gyrA locus shows a number of restriction site polymorphisms when compared with its counterpart in *E. coli* K-12 strain RW1053 (Fig. 1b). To isolate the 227 gene, chromosomal DNA was digested with BamHI and KpnI and DNA fragments in the 7- to 8-kb size range were purified by preparative gel electrophoresis. The DNA, now enriched for the gyrA gene, was ligated into BamHI-KpnI-digested pSP719, a 3.1-kb plasmid specifying ampicillin resistance and carrying multiple cloning sites. The DNA was used to transform *E. coli* DH5 (recA). Two independent size-selected libraries were obtained, each consisting of about 240 colonies. These libraries were probed by colony hybridization, using a randomly primed 32P-labeled SacI-HindIII probe from the 5′ end of the *E. coli* gyrA gene (Fig. 1). Two independent positive clones were obtained (Fig. 2) which contained identical plasmids with 7.5-kb inserts. Both plasmids had functional gyrA genes as demonstrated by their ability to confer temperature-independent growth on strain KKN453, which harbors a gyrAT(Ts) allele.

The frequency of gyrA clones in the two libraries was consistent with the calculated frequency of one positive clone per 160 colonies, using 7- to 8-kb size-selected DNA (25).

**Nucleotide sequence of the resistant gyrA gene.** The sequence of the gyrA gene in one plasmid, pMEC5, was determined by the Maxam-Gilbert chemical sequencing method, using a combination of 5′- and 3′-end-labeled DNA fragments (Fig. 3a and b). The 227 gene sequence is highly homologous to the *E. coli* K-12 gyrA sequence determined by Swanberg and Wang (32). The region 5′ to the gene has the same TATAAT box and ribosome-binding sequences previously noted in the wild-type K-12 gene. Following the stop codon, there is a perfect 9-bp inverted repeat which could act as a transcription termination signal.

The 2.6-kb gyrA gene from strain 227 encodes an 875-amino-acid protein. Within the coding region, there are 58 nucleotide changes compared with the K-12 gene, the vast majority (44 of 58) of which arise from C<–T (32 of 44) or G<–A (12 of 44) transitions. Only three nucleotide changes result in amino acid changes: the 227 protein has a tryptophan (Trp) residue instead of serine (Ser) at position 83, glutamate (Glu) for aspartate (Asp) at position 678, and serine for alanine (Ala) at position 828, arising from C-to-G, C-to-A, and G-to-T transversions, respectively.

Figure 4 compares *E. coli* and *Bacillus subtilis* gyrase A protein sequences (21, 32) in the regions around the three amino acid changes. The Trp residue replaces a Ser residue in a highly conserved part of the polypeptide chain close to the catalytic Tyr-122. The substitutions at positions 678 and 828 lie in less highly conserved regions. For *B. subtilis* gyrase A protein, a Glu residue is found at position 678, as is the case for the 227 protein.

**Construction and genetic analysis of chimeric gyrA genes.** To resolve which of the amino acid mutations in the 227 gyrA protein was responsible for its quinolone resistance properties, we constructed chimeric gyrA genes (Fig. 5) and examined their phenotypes (Table 2). By interchanging the smaller BamHI-Smal fragments between plasmids pMEC5 and pRM386 (which carries a wild-type gene), hybrid plasmids pMEC6 and pMEC7 were produced. The smaller BamHI-Smal fragment has a coding sequence that includes residue 83 of gyrA but not residues 678 and 828. Thus,
pMEC6 has a gyrA gene with serine at position 83 but changes at positions 678 and 828, while pMEC7 encodes a wild-type gyrA gene except for tryptophan at position 83 (Fig. 5). These plasmids were used to transform E. coli KNK453 gyrA(Ts) and DH5 gyrA96 recA. The properties of these strains are summarized in Table 2.

Strain KNK453 carries the gyrA43(Ts) allele and is viable at 37 but not at 42°C. First, we tested the quinolone resistance properties of KNK453 transformed with various plasmids at 37°C (Table 2). As expected, transformation with pRM386 carrying a susceptible gyrA gene did not affect the MICs of the various quinolones. However, pMEC5 conferred high-level resistance to nalidixic acid and resistance to enoxacin and ciprofloxacin, even at 37°C. The result shows that, in KNK453, quinolone-resistant gyrA genes on plasmids are dominant over the susceptible gyrA43(Ts) allele. A similar observation has been reported in E. coli KNK402, which carries the same gyrA43(Ts) allele (39). Although the molecular basis of this dominance is not understood, it enables direct comparison of MICs for transformed cells with the parental strain. At 37 or 42°C, cells transformed with pMEC6 exhibited a wild-type susceptibility to nalidixic acid. In contrast, pMEC7 behaved like pMEC5 in requiring high levels of drug for growth inhibition. Similar results were obtained for enoxacin and ciprofloxacin (Table 2). These results indicate that pMEC6 carries a quinolone-susceptible gyrA gene whereas the gyrA gene in pMEC7 is quinolone resistant. As pMEC7 carries the Ser-83→Trp change in gyrA, these observations establish that this mutation alone confers high-level quinolone resistance. Moreover, based on MIC comparisons (Tables 1 and 2), this mutation alone accounts for the resistance observed for strain 227.

We examined whether pMEC6 and pMEC7 are able to confer nalidixic acid susceptibility to the nalidixic acid-resistant strain DH5, which was originally selected for growth at 100 μg of the drug per ml. With the exception of the gyrA43(Ts) allele, it has been found that nalidixic acid-susceptible gyrA alleles are dominant over nalidixic acid-resistant ones (13). Thus, transformation of pRM386 into DH5 rendered the strain unable to grow at 50 μg of nalidixic acid per ml (results not shown). Presumably, the expression of some sensitive gyrase A protein in the cell is sufficient to allow the quinolone to exert its bactericidal effect. We tested the susceptibility of DH5 transformed with various plasmids constructs to nalidixic acid at 50 μg/ml. Plasmid pMEC6 was found to confer drug susceptibility to DH5, whereas cells transformed with pMEC7 remained resistant (results not shown). Therefore, pMEC6 and pMEC7 must carry nalidixic acid-susceptible and -resistant gyrA alleles, respectively.

**Quinolone-resistant properties of the 227 gyrase A protein.** Affinity-purified gyrase A protein from strain 227 comigrates on polyacrylamide gel electrophoresis with wild-type protein at an apparent molecular weight of 97,000 (Cullen et al., in press), in agreement with the actual molecular weight of 97,142 predicted from the 227 protein sequence (Fig. 5). We have shown previously that partially purified 227 gyrase A protein, when complemented with gyrase B subunit, generates a DNA supercoiling activity about 100-fold more resistant to enoxacin than wild-type gyrase. Thus, both genetic and protein approaches demonstrate that the 227 gyrase A protein is refractory to quinolone action.

It has been suggested that quinolones may exert their bactericidal effect through cellular processes other than changes in DNA supercoiling, e.g., by the formation of a gyrase-mediated double-stranded DNA break (4, 5). We have therefore examined whether gyrase reconstituted with highly purified strain 227 gyrase A protein can promote DNA cleavage. Gyrase-DNA complexes formed in the presence of enoxacin were treated with detergent to induce DNA cleav-
FIG. 3. DNA sequence analysis of the strain 227 gyrA gene. (a) DNA sequencing strategy. B, C, H, N, P, Pv, and S denote sites within the 227 gyrA gene for BglII, CiaI, HindIII, NcoI, PstI, PvuII, and ScaI, respectively. DNA restriction fragments, 32P labeled at these and other sites, were sequenced by the Maxam-Gilbert method (17). Sequence data were derived from both 5'-labeled (filled arrows) and 3'-labeled (open arrows) DNA ends. (b) Nucleotide sequence of the gyrA gene and the predicted protein sequence of the longest open reading frame. The 227 gyrA sequence is shown in full, while the K-12 gyrA sequence (32) is shown where different. Underlined are an upstream promoter motif (TATAATT) and a potential ribosome-binding site. A possible transcription termination signal is underlined just 3' of the gene. Asterisks denote changes in the protein sequence between the 227 and K-12 gyrase A subunits.
E. coli K12 76

E. coli 227 76

B. subtilis 671

E. coli K12 671

E. coli 227 671

B. subtilis 821

E. coli K12 821

E. coli 227 821

B. subtilis 837

FIG. 4. Sequence comparison of E. coli and B. subtilis gyrase A proteins. The protein sequence encompassing the three amino acid changes in strain 227 is shown in full. Sequences for the E. coli K-12 (32) and B. subtilis (21) proteins are shown where different.

age (6). Wild-type gyrase induced enoxacin-dependent linearization of supercoiled pBR322 DNA (Fig. 6, top lanes) with detectable DNA breakage at 0.5 μg of inhibitor per ml (lane c). Gyrase reconstituted with affinity-purified 227 gyrase A protein did not induce linearization even with 5 μg of enoxacin per ml (bottom wells, lane f). Mutation of the A subunit clearly affects the ability of gyrase to promote quinolone-dependent DNA cleavage.

DISCUSSION

We have cloned a gyrA gene from E. coli responsible for clinical resistance to 4-quinolone antibacterial agents. We find that a single mutation of Ser-83→Trp in the 875-amino-acid gyrase A protein is sufficient to confer high-level bacterial resistance. These results are the first molecular analysis of quinolone resistance in a clinical isolate. By examining the properties of chimeric gyrA genes, we could show that two other amino acid substitutions in the gyrase A protein from the clinical strain, Asp-678→Glu and Ala-828→Ser, were neutral changes and did not contribute to the resistance phenotype. Clearly, the construction and analysis of chimeric genes constitute a powerful tool for determining for clinical isolates whether multiple mutations arise simply from strain differences or from stepwise selection for resistance during anti-infective treatment. The examination of chimeric genes is particularly useful for clinical isolates when the parental susceptible strain is often unavailable. It appears that, for uropathogenic strain 227, enoxacin therapy has selected a single-step mutation, rendering DNA gyrase resistant to the inhibitory effects of quinolones.

The studies reported here on the clinically resistant gyrA gene and gyrase A protein complement very recent work on bacterial resistance.}

**TABLE 2.** High-level quinolone resistance conferred by a single point mutation

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>MIC (μg/ml) determined at 37°C</th>
<th>Nalidixic acid</th>
<th>Enoxacin</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNK453</td>
<td>4</td>
<td>0.25</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>KNK453(PR386)</td>
<td>8 (8)</td>
<td>0.25 (0.25)</td>
<td>0.03</td>
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<tr>
<td>KNK453(pMEC5)</td>
<td>250 (&gt;125)</td>
<td>4 (&gt;1)</td>
<td>0.50</td>
<td></td>
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<tr>
<td>KNK453(pMEC6)</td>
<td>4 (4)</td>
<td>0.25 (0.25)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>KNK453(pMEC7)</td>
<td>500 (1,000)</td>
<td>2 (2)</td>
<td>0.50</td>
<td></td>
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</tbody>
</table>

* Value in parentheses is MIC determined at 42°C.

**FIG. 5.** Construction of chimeric gyrA genes. Plasmids pMEC5 and pRM386 carry drug-resistant and drug-susceptible gyrA alleles, indicated by filled and open boxes, respectively. Arrows denote transcription start sites. B, H, K, and Sm denote sites for BamHI, HindIII, KpnI, and Smal, respectively. These plasmids were each digested with BamHI and Smal, and the resulting fragments were isolated and purified. Ligation was performed in which the smaller BamHI-Smal fragments were exchanged to produce the heterologous plasmids pMEC6 and pMEC7.

**FIG. 6.** Evidence that strain 227 gyrase A protein does not promote quinolone-dependent DNA breakage. Purified gyrase A protein (25 μg) from quinolone-susceptible strain N4186 (top wells) or 227 (bottom wells) was incubated with gyrase B subunit (25 μg) and 0.6 μg of supercoiled pBR322 DNA in the absence (lane a), or presence of enoxacin at 0.2, 0.5, 1, 2, and 5 μg/ml (lanes b to f, respectively). Samples were treated with sodium dodecyl sulfate and proteinase K and electrophoresed in a 0.8% agarose gel. Lane g contains untreated relaxed pBR322 DNA. Arrow indicates position of linear pBR322 DNA.
quino
lone mutations in E. coli K16 selected in the laboratory by one-step exposure to nalidixic acid or pipemidic acid (39). Although these authors did not characterize the gyrase A protein, they showed that resistance can arise by mutation at several loci in the gyrA gene. They found in two instances that high-level laboratory resistance to nalidixic acid or pipemidic acid arose by mutation of Ser-83 to leucine or tryptophan, respectively. Mutation of Gln-106—His and Ala-67→Ser resulted in low-level laboratory resistance to nalidixic acid and pipemidic acid, respectively. It is intriguing that clinical resistance to enoxacin during anti-infective therapy and laboratory selection for high-level pipemidic acid resistance both arise from substitution of the same residue in the gyrase A protein.

How might residue 83 participate in quinolone action? Two models may be considered that account for inhibition of gyrase by quinolones. First, the drugs could bind directly to the gyrase complex at a site adjacent to the catalytic Tyr-122 residue of the gyrase A subunit. In an alternate model, Shen and Perret (27) have suggested, based on binding studies, that DNA and not gyrase is the target for quinolones. They envisage that there exist a few high-affinity quinolone-binding sites on DNA. Gyrase would then recognize and bind to these drug-DNA adducts. In a further elaboration of this model (cited in reference 5), Shen suggests that the quinolone binds the single-stranded DNA region formed by the transient staggered 4-bp break introduced by the gyrase A subunits. Evidence is conflicting as to whether quinolones do or do not bind to DNA (24, 27, 33). Moreover, DNase I footprinting experiments and other data show that gyrase can bind and cleave DNA even in the absence of quinolones (6, 7).

Whichever model applies, the clustering of quinolone resistance mutations in a highly conserved region of the gyrase A polypeptide adjacent to Tyr-122 could suggest a binding site for the quinolone. The gyrase B subunit could also participate, as Yamagishi et al. (38) have mapped and sequenced two mutations in the carboxy-terminal end of gyrase B protein, which resemble the carboxy-terminal end of the gyrase A subunit that confer low-level quinolone resistance. There are conceivably two quinolone-binding sites in the symmetrical A1B2 complex involving A2B2 subunit interfaces, consistent with the ability of quinolones to mediate both single- and double-stranded DNA breaks (28). It is tempting to suggest that mutations in either the gyrase A or B proteins could then interfere with quinolone binding. By reducing drug binding to the gyrase complex, the interfering effects of quinolones on DNA supercoiling must then be observed. We note in this regard that the tryptophan side chain introduced by mutation of Ser-83 bears some similarity in size to the ring system of a quinolone and might therefore be particularly effective in impeding drug binding.

It is not known whether these simple steric blocking ideas or more complicated models involving conformational alteration of the gyrase complex actually account for quinolone resistance. Clearly, structural and biophysical studies on quinolone-resistant gyrase complexes are necessary to resolve this issue. Quinolone-resistant gyrase A proteins have been purified from a number of clinically resistant bacterial species (1, 26; unpublished results). It remains to be determined whether mutation of Ser-83 in the E. coli gyrA subunit (or its functional equivalent in other bacterial species) is a common feature of resistance.

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LITERATURE CITED


