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 A₂B₂ 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*(Ts) 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 A₂B₂ 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^r) (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 ceftazidime (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]dATP (each 3,000 Ci/mmol), T4 polynucleotide kinase, restriction enzymes, Hybond-N, and Multiprime 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 proteinase 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 *Nco*I were from New England Bio-

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Labs, Inc., Beverly, Mass. Low-gelling 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 2×10^4 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 *SacI* 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 *SacI*-*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 *SacI* and *HindIII* sites internal to the *gyrA* gene.

DNA cloning of the strain 227 *gyrA* gene. Genomic DNA was digested with *Bam*HI and *Kpn*I, 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 *Bam*HI-*Kpn*I-cut plasmid pSPT19 and used to transform highly competent *E. coli* DH5 cells, which were then plated on L-ampicillin plates (25 μ g/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 μ g/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 *SacI*-*HindIII* fragment from the 5' end of the K-12 *gyrA* gene (Fig. 1) excised from plasmid pRM386 and labeled with [α - 32 P] 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 mM MgCl₂–6 mM 2-mercaptoethanol (15 μ l), using [α - 32 P]dATP (15 μ Ci) 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 *Bgl*II ends, dGTP (50 μ M) 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 *Bam*HI, *Cla*I, *Hind*III, *Kpn*I, and *Sac*I, respectively. The *gyrA* gene was localized and restriction maps were determined by Southern blotting of appropriately digested chromosomal DNA, using 600-bp *SacI*-*Hind*III and 2.8-kb *Hind*III 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., *Bgl*II, *Hind*III, *Cla*I, *Pvu*I, *Nco*I, and *Sac*I. 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 *SacI*-*Nru*I fragment, a 900-bp *Bgl*II fragment, a 600-bp *SacI*-*Hind*III fragment, and a 2.0-kb *Bam*HI-*Sac*I fragment containing the 5' region of the gene. When appropriate, these fragments were 5' labeled at *Hin*fI, *Taq*I, *Msp*I, *Rsa*I, or *Hae*III 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 *Bam*HI and *Sma*I, and each pair of fragments were separated and purified from low-gelling agarose. Heterologous pairs of *Bam*HI-*Sma*I 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 μ g of drug per ml at 37°C.

Purification of gyrase A protein and DNA breakage assays. For use in DNA cleavage experiments, strain 227 gyrase A 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 Polymin P and ammonium sulfate precipitation. Protein from

TABLE 1. Quinolone resistance properties of *E. coli* clinical isolate 227 compared with RW1053, a susceptible K-12 strain

Strain	MIC ($\mu\text{g/ml}$) ^a							Gen
	Nal	Nor	Ofi	Pef	Eno	Cip	Cef	
RW1053	4	0.03	0.015	0.03	0.03	0.004	0.13	1
227	500	1	1	1	2	0.25	0.25	2

^a Nal, Nalidixic acid; Nor, norfloxacin; Ofi, ofloxacin; Pef, pefloxacin; Eno, enoxacin; Cip, ciprofloxacin; Cef, ceftazidime; Gen, gentamicin.

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 novobiocin-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. McEachern, 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 radiolabeled 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 digests 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 pSPT19, 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 KNK453, which harbors a *gyrA*(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 (23).

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 $\leftrightarrow$ T (32 of 44) or G $\leftrightarrow$ 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-SmaI* fragments between plasmids pMEC5 and pRM386 (which carries a wild-type gene), hybrid plasmids pMEC6 and pMEC7 were produced. The smaller *BamHI-SmaI* fragment has a coding sequence that includes residue 83 of *gyrA* but not residues 678 and 828. Thus,

FIG. 2. Isolation of plasmid clones carrying the *gyrA* gene from *E. coli* 227. Two independent plasmid libraries of 227 DNA were screened by colony hybridization, using a *SacI-HindIII* *gyrA* probe (see legend to Fig. 1). Strongly hybridizing colonies on each filter are indicated by arrows. All colonies show some hybridization to the probe arising from their chromosomal *gyrA* genes.

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 plasmid 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. These genetic studies show unequivocally that it is the Ser-83→Trp mutation rather than changes at either 678 or 828 that renders the gyrase A protein resistant to quinolones.

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. 3). 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-

<i>E. coli</i> K12	76	- - - - - Ser - - - - -	92
<i>E. coli</i> 227		LysTyrHisProHisGlyAspTrpAlaValTyrAspThrIleValArgMet	
<i>B. subtilis</i>		- - - - - Ser - - - - - GluSerMet - - -	
<i>E. coli</i> K12	671	- - - - - Asp - - - - -	687
<i>E. coli</i> 227		LysValAlaIleLysLeuValGluGlyAspGluLeuIleGlyValAspLeu	
<i>B. subtilis</i>		LeuIle - LeuSer - ArgGluAsp - - - Met - - Arg -	
<i>E. coli</i> K12	821	- - - - - Ala - - - - -	837
<i>E. coli</i> 227		GlyValIleLeuIleArgThrSerGluAspGluAsnValValGlyLeuGln	
<i>B. subtilis</i>		- - Arg - - - MetAla - Glu - His - AlaThrValAla	

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 μ g of inhibitor per ml (lane c). Gyrase reconstituted with affinity-purified 227 gyrase A protein did not induce linearization even with 5 μ g 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

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 *Bam*HI, *Hind*III, *Kpn*I, and *Sma*I, respectively. These plasmids were each digested with *Bam*HI and *Sma*I, and the resulting fragments were isolated and purified. Ligations were performed in which the smaller *Bam*HI-*Sma*I fragments were exchanged to produce the heterologous plasmids pMEC6 and pMEC7.

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

Strain (plasmid)	MIC (μ g/ml) determined at 37°C ^a		
	Nalidixic acid	Enoxacin	Ciprofloxacin
KNK453	4	0.25	0.03
KNK453(pRM386)	8 (8)	0.25 (0.25)	0.03
KNK453(pMEC5)	250 (>125)	4 (>1)	0.50
KNK453(pMEC6)	4 (4)	0.25 (0.25)	0.03
KNK453(pMEC7)	500 (1,000)	2 (2)	0.50

^a Value in parentheses is MIC determined at 42°C.

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

FIG. 6. Evidence that strain 227 gyrase A protein does not promote quinolone-dependent DNA breakage. Purified gyrase A protein (25 U) from quinolone-susceptible strain N4186 (top wells) or 227 (bottom wells) was incubated with gyrase B subunit (25 U) and 0.6 μ g of supercoiled pBR322 DNA in the absence (lane a) or presence of enoxacin at 0.2, 0.5, 1, 2, and 5 μ g/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.

quinolone mutations in *E. coli* KL16 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 or nalidixic 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 Pernet (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 that confer low-level quinolone resistance. There are conceivably two quinolone-binding sites in the symmetrical A₂B₂ complex involving A-B 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 scission might then be obviated. 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* gyrase A subunit (or its functional equivalent in other bacterial species) is a common feature of resistance.

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