Detection of *gyrA* and *gyrB* Mutations in Quinolone-Resistant Clinical Isolates of *Escherichia coli* by Single-Strand Conformational Polymorphism Analysis and Determination of Levels of Resistance Conferred by Two Different Single *gyrA* Mutations

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Twelve quinolone-resistant clinical isolates of *Escherichia coli* (nalidixic acid MICs, 64 to 512 μg/ml; norfloxacin MICs, 0.25 to 8 μg/ml) were transformed with plasmid pJSW101 carrying the *gyrA*+ gene and with plasmid pJB11 carrying the *gyrB*+ gene to examine the proportion of *gyrA* and *gyrB* mutations. Transformation with pJSW101 resulted in complementation (nalidixic acid MICs, 4 to 32 μg/ml; norfloxacin MICs, 0.06 to 0.25 μg/ml). In contrast, no change in MICs were observed after transformation with pJB11. A 418-bp fragment of *gyrA* from the 12 strains was amplified by PCR. Direct DNA sequencing of that fragment identified the causes of quinolone resistance in eight strains as a single point mutation leading to a substitution of the serine at position 83 (Ser-83) to Leu and in four strains as a single point mutation leading to a substitution of Asp-87 to Gly. Exchange of the fragment from one of these strains with that of *gyrA*+ and transformation of resistance with the hybrid *gyrA* plasmid indicated the contribution of Gly-87 to resistance and the stabilities of mutants containing GyrA (Gly-87). Thus, *gyrA* gene mutations are probably encountered more often than *gyrB* gene mutations in clinical isolates of *E. coli*. In addition, the substitution of Asp-87 to Gly can be encountered in such strains. On the basis of the level of resistance found in the fragment exchange experiment, the quinolone resistance attributable to Gly-87 appears to be comparable to that attributable to Leu-83. The levels of resistance found in the clinical isolates shown to have a Gly-87 mutation (nalidixic acid MICs, 64 to 512 μg/ml; norfloxacin MICs, 0.5 to 4 μg/ml) suggest that the Gly-87 mutation causes resistance at the level of the nalidixic acid MIC (64 μg/ml) or the norfloxacin MIC (0.5 μg/ml or less) and that the additional increments in resistance seen in the other strains with higher levels of resistance may be attributable to additional mutations. The single-strand conformational polymorphism analysis with PCR products readily detected the Leu-83 and Gly-87 mutations.

The mechanisms of bacterial resistance to quinolones in gram-negative bacteria include chromosomal mutations that either alter DNA gyrase or reduce membrane permeation (15). Two types of gyrase mutants have been described: mutations in the *gyrA* gene at 48 min and mutations in the *gyrB* gene at 83 min on the *Escherichia coli* chromosome (4, 8, 21, 22). Several types of permeability mutations were characterized; in addition to the *nalB* mutation, *nfxB*, *norC*, and several mutations selected with quinolones (*nfxC*, *cfxB*) or with tetracycline or chloramphenicol (*marA*) encode an altered MarR repressor protein (2, 3, 9–11).

Several methods have been used to identify alterations in the *gyrA* and *gyrB* genes associated with quinolone resistance. These have included cloning of the *gyrA* and *gyrB* genes from resistant strains and identification of the changes in the nucleotide sequences as well as purification of the GyrA and GyrB proteins to demonstrate drug resistance as a result of enzyme activity conferred by one subunit in a mixing experiment. Certain common *gyrA* mutations also abolish a *Hin*fI restriction enzyme site and may be detected as a restriction fragment length polymorphism (6). A popular simpler method involves transformation of a resistant strain with a plasmid containing

the wild-type *gyrA*+ gene. Because of the dominance of *gyrA*+ over *gyrA* alleles for resistance, such merodiploid transformed strains become more quinolone susceptible.

The frequency of *gyrA* and *gyrB* mutations among clinical isolates of gram-negative rods has only been studied by Nakamura et al. (12) and Yoshida et al. (25). In the present study, we evaluated the relative occurrence of *gyrA* and *gyrB* mutations in quinolone-resistant clinical isolates of *E. coli* by transformation with plasmids carrying the wild-type genes and measuring the MICs of different quinolones for the transformants. DNA amplification by PCR, single-strand conformational polymorphism analysis (SSCP), and direct DNA sequencing were then used to identify the mutations. A fragment exchange procedure was used to prove the contribution of Asp to Gly substitution at position 87 (Asp-87 to Gly-87) of the GyrA protein to resistance and the stability of the mutant.

MATERIALS AND METHODS

Bacterial strains. Twelve ampicillin-susceptible and quinolone-resistant *E. coli* clinical isolates were used for the study; these strains were isolated from patients with urinary tract infections (*n* = 8), blood cultures (*n* = 3), and a cerebrospinal fluid sample (*n* = 1). *E. coli* KL16, which is susceptible to quinolones; KF130 *gyrA*, a mutant derived from KL16; and N-24 *nalD* and N-31 *nalC*, both of which harbor a mutation in the *gyrB* gene, were used as controls. Two quinolone-susceptible clinical isolates were included for SSCP analysis. *E. coli* DH10B was

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used as a recipient strain for transformation with recombinant plasmids, and *E. coli* KNK453 *gyrA*(Ts) was used to check temperature complementation and resistance after transformation with plasmids isolated from DH10B.

trans-Complementation plasmids pJSW101 (*gyrA*⁺) and pJB11 (*gyrB*⁺). Plasmid pJSW101 (5.5 kb) is derived from pUC19 by ligation on the *Bam*HI and *Sal*I sites of the wild-type *gyrA* gene isolated from strain KL16 (18). pJB11 (6.3 kb) is derived from pBR322 by ligation on *Ava*I and *Eco*RI sites of the wild-type *gyrB* gene isolated from KL16 (23). Transformation was performed by electroporation (5), and transformants were selected on Luria-Bertani agar containing ampicillin at 100 µg/ml to ensure the presence of the complementing plasmid (18).

Antimicrobial agents. Nalidixic acid was a gift from Winthrop, norfloxacin was from Merck Sharp & Dohme Research Laboratories, pefloxacin was from Bellon, and ciprofloxacin was from Miles Pharmaceuticals. Sodium ampicillin was purchased from Sigma Chemical Co.

Determination of MICs. MICs of various quinolones were determined by the agar dilution method with Mueller-Hinton agar and an inoculum of 10^6 cells per ml.

PCR experiments. A 418-bp *gyrA* fragment from *E. coli* strains was amplified by PCR. For each strain, chromosomal DNA was prepared from 5 µl of an overnight culture diluted into 52 µl of sterile water, and the mixture was boiled for 3 min. DNA was subjected to PCR by using two 24-mer oligonucleotide primers, 5′-GTGGATCCTATATCAGGCATTGGA and 5′-TCGAATTCCGT CGATAGAACCGAA, the sequences of which are identical to nucleotide positions −110 to −95 and complementary to positions +325 to +341, respectively, of the *E. coli* K-12 *gyrA* gene (19).

Reactions were performed in a DNA thermal cycler (Perkin-Elmer Cetus) as described previously (18).

SSCP analysis. Aliquots of 1 µl of PCR products were mixed with 1 µl of denaturant solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), and the mixture was heated at 100°C for 4 min and then cooled on ice. The single-stranded PCR products were then separated by polyacrylamide gel (12.5%) electrophoresis at 15°C by using the Phast system apparatus (Pharmacia). DNA bands were then visualized by silver staining. The gel was prerun at 400 V, 5 mA, and 1 W for 30 V-h. Samples were applied at 25 V, 5 mA, and 1 W for 2 V-h. Samples were run at 400 V, 5 mA, and 1 W for 750 V-h.

DNA sequencing. PCR-amplified *gyrA* fragments from clinical isolates were purified by using the Miniprep Spun columns (Pharmacia) and were then directly sequenced by the dideoxy-chain termination method (17) with the T7 sequencing kit (Pharmacia) and [³⁵S]dATP (Amersham). The oligonucleotides used for PCR were also used as primers for DNA sequencing. An additional oligonucleotide, 5′-ATTGTTGGCCGTGCGCTGCCAGAT-3′ from positions +85 to +108 in *gyrA* (19), was also used as the primer in the sequencing reactions.

Construction of hybrid plasmids and transformation. The 418-bp *gyrA* fragment containing part of the *gyrA* gene from *gyrA* mutant KF130 or clinical isolate BER was exchanged with the same fragment of the *gyrA* gene from KL16 in plasmid pJSW101. The ligation mixtures were used to transform DH10B with ampicillin as a selection agent (18).

The plasmids were isolated from DH10B by the Magic Minipreps procedure (Promega, Madison, Wis.) and were used to transform KNK453 (Ts); this was followed by selection for ampicillin resistance at 30°C. Plasmid-encoded *gyrA* alleles for resistance have been shown to express quinolone resistance when the chromosomal *gyrA* allele for susceptibility is conditionally defective (4, 24). The transformants were tested for quinolone resistance and temperature complementation.

RESULTS

Antimicrobial susceptibility. All of the clinical isolates studied were highly resistant to nalidixic acid (MICs, 64 to 512 µg/ml) but had only a low level of resistance to fluoroquinolones (pefloxacin MICs, 1 to 16 µg/ml; norfloxacin MICs, 0.25 to 8 µg/ml; ciprofloxacin MICs, 0.06 to 4 µg/ml), and the MICs of the four drugs for the indicated strains were as follows: KL16, 4, 0.12, 0.06, and 0.01 µg/ml, respectively; KF130, 128, 1, 0.5, and 0.16 µg/ml, respectively; N-24, 64, 0.5, 0.25, and 0.063, µg/ml, respectively; N-31, 64, 0.03, 0.016, and 0.004 µg/ml, respectively.

trans-Complementation tests. No significant change in MICs was observed after transformation of either KF130 *gyrA* or clinical isolates with pUC19. After transformation with pJSW101, the MICs for all of these strains demonstrated substantial reductions (Table 1). No significant change was observed after transformation of clinical isolates with pJB11 and pBR322 (Table 2). The *gyrB* complementation test was, however, positive for the control strains N-24 *nalD* and N-31 *nalC*. For N-31 *nalC*, a mutant resistant to nalidixic acid and hyper-

susceptible to fluoroquinolones, the decrease was a factor of 8 for nalidixic acid, whereas no change was observed for ciprofloxacin and slight increases in pefloxacin and norfloxacin MICs were observed.

Nucleotide sequence analysis. As shown in Fig. 1, *gyrA* mutant KF130 as well as eight clinical isolates were characterized by a C-to-T mutation at codon 83 of *gyrA*, leading to a Ser-to-Leu substitution, and the four other mutants were characterized by A-to-G mutations at codon 87 leading to Asp-to-Gly substitutions. The C-to-T transition at codon 85, resulting in no amino acid change, was found in the four mutants with Asp-87 to Gly-87 changes, including BER, and in seven mutants with Ser-83 to Leu-83 changes, including HM72, but not DOU. No other nucleotide changes were found in the region between codons 60 and 110, which was sequenced (Fig. 1).

trans-Complementation tests with chimeric *gyrA* gene. To confirm that the A-to-G mutation was responsible for the quinolone resistance of BER, we used a fragment exchange procedure in which a 418-bp fragment of *gyrA* (nucleotides −110 to +308) from strain BER was ligated in frame into the remainder (nucleotides +309 to +2625) of wild-type *gyrA*⁺ in plasmid pJSW101 to create plasmid pBER. Similar hybrid plasmids containing the 418-bp *gyrA* fragment from KL16(pKL16) and KF130(pKF130) have been reported previously (18). To assess the effects of the hybrid plasmids harboring the mutated *gyrA* gene of BER, we transformed KNK453 *gyrA*(Ts). The MICs of the quinolone for KNK453(pKL16) and KNK453 were the

TABLE 1. Complementation of quinolone resistance by plasmid-encoded *gyrA*⁺

Isolate	Quinolone MIC decrease[a]			
	NAL	PFX	NFX	CFX
Clinical isolates (pJSW101)	64 (16–128)	32 (8–64)	16 (2–128)	64 (4–512)
KF130 *gyrA*	32	8	4	8
Clinical isolates (pUC19)	2 (1–4)	2 (1–4)	1 (1–4)	1 (1–4)
KF130 *gyrA* (pUC19)	2	2	1	2

[a] Values are means (ranges). NAL, nalidixic acid; PFX, pefloxacin; NFX, norfloxacin; CFX, ciprofloxacin.

TABLE 2. Complementation of quinolone resistance by plasmid-encoded *gyrB*⁺

Isolate	Quinolone MIC decrease[a]			
	NAL	PFX	NFX	CFX
Clinical isolates (pJB11)	2 (0.5–4)	2 (0.5–4)	2 (0.5–4)	2 (0.5–4)
N-24 *nalD* (pJB11)	8	8	4	8
N-31 *nalC* (pJB11)	8	0.5	0.5	1
Clinical isolates (pBR322)	2 (1–4)	2 (0.5–4)	2 (0.5–4)	2 (1–4)
N-24 *nalD* (pBR322)	2	2	2	2
N-31 *nalC* (pBR322)	1	2	1	1

[a] Values are means (ranges). NAL, nalidixic acid; PFX, pefloxacin; NFX, norfloxacin; CFX, ciprofloxacin.

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                  83           8 5        87

KL16:   5'--GGT GAC TCG GCG GTC TAT GAC ACG ATG--3'

        Gly  Asp  Ser  Ala  Val  Tyr  Asp  Thr  Ile

KF130:  5'--GGT GAC TTG GCG GTC TAT GAC ACG ATC--3'

        Gly  Asp  Leu  Ala  Val  Tyr  Asp  Thr  Ile

HM72:   5'--GGT GAC TTG GCG GTT TAT GAC ACG ATC--3'

        Gly  Asp  Leu  Ala  Val  Tyr  Asp  Thr  Ile

BER  :  5'--GGT GAC TCG GCG GTT TAT GGC ACG ATC--3'

        Gly  Asp  Ser  Ala  Val  Tyr  Gly  Thr  Ile

DOU  :  5'--GGT GAC TTG GCG GTC TAT GAC ACG ATC--3'

        Gly  Asp  Leu  Ala  Val  Tyr  Asp  Thr  Ile
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FIG. 1. Partial nucleotide sequences of KL16, KF130, HM72, BER, and DOU. The amino acid differences at codons 83 and 87 are indicated in boldface type, denoting the region in which resistance mutations have been found.
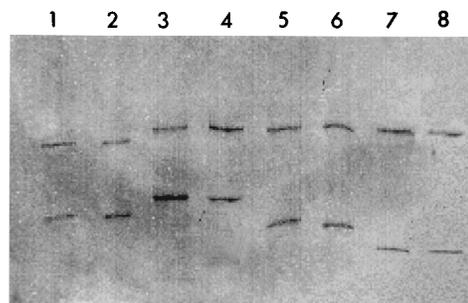

FIG. 2. PCR-SSCP analysis of 418 bp in the *gyrA* gene of eight strains of *E. coli* by using the PhastGel (12.5%) homogeneous system. Lane 1, wild type (KL16); lane 2, quinolone-susceptible clinical isolate; lanes 3 and 4, mutants with Asp→Gly change at codon 87 and Val→Val change at codon 85 (lane 3, strain BER); lanes 5 and 6, mutants with Ser→Leu change at codon 83 (lane 5, strain KF130; lane 6, strain DOU); lanes 7 and 8, mutants with Ser→Leu change at codon 83 and Val→Val change at codon 85 (lane 7, strain HM72).

same (Table 3). For KNK453(pKF130) and KNK453(pBER), the increment of the MICs was 32- and 16-fold for nalidixic acid and 4- to 8-fold for fluoroquinolones.

SSCP analysis of a PCR-amplified 418-bp *gyrA* fragment. Another method for detecting mutations is analysis of SSCP patterns. This method has, however, only been applied in a limited manner for the detection of *gyrA* mutations (26). The SSCP analysis with PCR products from the isolates displayed four patterns with different mobilities (Fig. 2). The susceptible strains showed identical migration patterns, but the resistant strains displayed three different migration patterns. All strains with an Asp-87 (GGC) mutation and a silent mutation at codon 85, including BER, had identical patterns. Strains with a Ser-83 (TTG) mutation exhibited two additional distinct SSCP patterns: one was identical to that of KF130 (mutant DOU); the other, which was different from that of KF130, was observed with mutants harboring an additional silent mutation at codon 85.

DISCUSSION

All 12 clinical *E. coli* isolates tested were found to have *gyrA* mutations by the plasmid complementation test and DNA sequencing. No strains had evidence of a *gyrB* mutation by the plasmid complementation test. In addition, other mechanisms could contribute to quinolone resistance in these strains because the complementation by plasmid-encoded *gyrA*⁺ was incomplete. Nakamura et al. (12) found that five strains had a

gyrA mutation, one isolate had a *gyrB* mutation, and two isolates had unidentified mutations. Particularly notable has been the occurrence of alterations at position 83 of the GyrA protein (13).

DNA sequencing identified in eight of our strains Ser-to-Leu changes at codon 83 of the *gyrA* gene. In the other four strains, we have found Asp-to-Gly changes at codon 87 of the *gyrA* gene, a novel single mutation which has not been reported until now among clinical isolates. Recently, Heisig et al. (7) and Vila et al. (20) have described double mutants (Ser-83 and Asp-87), but in our strains with the mutations at codon 87, there was no concomitant mutation at codon 83. At position 87, two mutations have been identified previously in *E. coli*, one in a clinical isolate (Asp-87 to Val-87) (13) and the other in a spontaneous mutant (Asp-87 to Asn-87) (24). The Asp-87 to Gly-87 mutation was also identified in spontaneous fluoroquinolone-resistant mutants of *Mycobacterium smegmatis* (16).

Amplification of a 418-bp fragment of the *gyrA* gene known to encompass the region of quinolone-resistant mutations and the exchange of this fragment within the wild-type *gyrA* gene showed that the resistance determinant of BER was located in this region. The level of resistance attributable to the substitution Asp-87 to Gly-87 appears to be comparable to that attributable to the substitution Ser-83 to Leu-83 and similar to that previously attributed to the Asp-87 to Asn-87 substitution (24). SSCP analysis readily detected the Leu-83 and Gly-87 mutations in our strains. A distinct and consistent pattern was seen for all four Gly-87 mutants. One strain harboring the Ser-to-Leu mutation at codon 83 showed a pattern identical to that from KF130. The seven other strains exhibited the same SSCP pattern, which was different from that of KF130. This difference is not known, but it might be explained by the presence of the additional silent mutation at codon 85. SSCP analysis is a simple, sensitive method for the detection of nucleotide sequence changes in DNA. Since its description by Orita et al. (14), it has been used to identify mutations in the *rpoB* gene of *Mycobacterium tuberculosis* (1) and the *gyrA* gene of *Staphylococcus aureus* (26). This technique detected all Leu-83 and Gly-87 mutations that we identified by DNA sequencing. Heterogeneity seen in the SSCP pattern for Ser-83 mutations, however, indicated that mutations inferred from SSCP patterns should be confirmed by DNA sequencing. Further studies are needed to determine the ability of SSCP analysis to detect other less common *gyrA* mutations, but our data suggest that SSCP analysis may be a useful screening method for identifying common *gyrA* mutations in clinical isolates of *E. coli*.

TABLE 3. Effects of *gyrA* plasmids on temperature sensitivity and quinolone resistance of KNK453 [*gyrA*(Ts)]

Strain (plasmid)	Growth at 43°C[a]	MIC (µg/ml) 30°C[b]		
		NAL	NFX	CFX
KNK453	−	8	0.12	0.02
KNK453(pKL16)	+	8	0.12	0.02
KNK453(pKF130)	+	256	0.5	0.16
KNK453(pHM72)	+	256	0.5	0.16
KNK453(pBER)	+	128	0.5	0.16

[a] +, presence of growth; −, absence of growth.
[b] NAL, nalidixic acid; NFX, norfloxacin; CFX, ciprofloxacin.

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