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A Novel, Double Mutation in DNA Gyrase A of *Escherichia coli* Conferring Resistance to Quinolone Antibiotics

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A spontaneous *Escherichia coli* mutant, named Q_3, resistant to nalidixic acid was obtained from a previously described clinical isolate of *E. coli*, Q_2, resistant to fluoroquinolones but susceptible to nalidixic acid (E. Cambau, F. Bordon, E. Collatz, and L. Gutmann, Antimicrob. Agents Chemother. 37:1247–1252, 1993). Q_3 harbored the mutation Asp82Gly in addition to the Gly81Asp mutation of Q_2. The different mutations leading to Gly81Asp, Asp82Gly, and Gly81AspAsp82Gly were introduced into the *gyrA* gene harbored on plasmid pJSW102, and the resulting plasmids were introduced into *E. coli* KNK453 (*gyrA*^{ts}) by transformation. The presence of Asp82Gly or Gly81Asp alone led to a low-level resistance to fluoroquinolones but not to nalidixic acid resistance. When both mutations were present, resistance to both nalidixic acid and fluoroquinolones was expressed. Purified gyrases of the different mutants showed similar rates of supercoiling. Dominance of the various *gyrA* mutant alleles harbored on plasmids was examined. The susceptibility to quinolones associated with wild-type *gyrA* was always dominant. The susceptibility to nalidixic acid expressed by the Gly81Asp mutant was dominant, while that expressed by the Asp82Gly mutant was recessive. From these results, we hypothesize that some amino acids within the quinolone resistance-determining region of gyrase A are more important for the association of subunits rather than for the activity of the holoenzyme.

The quinolones are a clinically useful group of antibacterial agents (7, 31) for which the molecular target in *Escherichia coli* is thought to be DNA gyrase, the bacterial enzyme which supercoils DNA using the energy of ATP hydrolysis (11, 18, 24). DNA gyrase from *E. coli* consists of two subunits, A and B, with molecular masses of 97 and 90 kDa, respectively. The active enzyme is the A_2B_2 tetramer. The mechanisms of bacterial resistance to quinolones in gram-negative bacteria include chromosomal mutations that either alter DNA gyrase and topoisomerase IV, increase efflux of quinolones, or reduce levels of quinolone accumulation in the cells (8, 10, 17, 20, 35–39). Mutations in the *gyrA* gene of *E. coli* involved in resistance are clustered in a region between nucleotides 199 (Ala67) and 318 (Gln106), designated the quinolone resistance-determining region (QRDR) (9, 16), and most of the quinolone-resistant clinical isolates of *E. coli* have a substitution at Ser83 (9, 13, 16, 29, 38). The resistance patterns associated with *gyrA* mutations are generally characterized by higher MICs of nalidixic acid (8- to 400-fold increase in the MICs) than those observed for the newer fluoroquinolones (17, 35, 36).

We previously described a clinical isolate, *E. coli* Q_2, which expressed high-level resistance to fluoroquinolones but remained susceptible to nalidixic acid (6, 27). Two mechanisms were involved in this peculiar pattern of resistance: one was an altered gyrase due to a transition from G to A at position 242 of the *gyrA* gene, leading to a Gly81Asp substitution in the gyrase A subunit, while the other was an impaired accumulation. Experiments designed to obtain further insight into the altered function of mutant gyrases led us to study a nalidixic acid-resistant derivative of Q_2.

The results of this study were presented, in part, previously (34).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the present study are listed in the Table 1. All *E. coli* strains were grown in brain heart infusion (BHI) broth or agar. A spontaneous nalidixic acid-resistant mutant of Q_2, called Q_3, was selected in vitro by plating *E. coli* Q_2 on BHI agar containing nalidixic acid in increasing concentrations. Plasmid DNA was prepared by the alkaline lysis method of Birnboim and Doly (4).

Antibiotics and MICs. Nalidixic acid was provided by Sterling-Winthrop, Clichy, France, and ciprofloxacin was provided by Bayer Pharma, Sens, France. The MICs of the quinolones were determined on BHI agar plates by using a Steers replicator device that delivered ca. 10^4 bacteria per spot. MICs were read after 18 h of incubation at 37°C.

PCR amplification of chromosomal DNA from *E. coli* Q_3 and DNA sequencing. A 668-bp fragment including the QRDR of *gyrA* was amplified from the chromosomal DNA of Q_3 by PCR with oligonucleotide primers I (5′-GAGGAAGA GCTGAAGAGCTCCT-3′) and II (5′-CCGGTACGGTAAGCTTCTTCAA-3′) (Bioprobe System, Montreuil-sous-bois, France). Primer I corresponds to nucleotide positions 40 to 61, and primer II is complementary to positions 686 to 707 of the *E. coli* K-12 *gyrA* gene (6). PCR was carried out with *Taq* polymerase from Boehringer, Mannheim, Germany, as recommended by the manufacturer. The reaction mixture contained 2.5 U of *Taq* polymerase per 100 μl of reaction mixture; 50 ng of DNA template; Tris-HCl (10 mM; pH 8); KCl (50 mM); $MgCl_2$ (1.5 mM); gelatin (0.1%); 200 μM each dATP, dCTP, dGTP, and dTTP; and the primers at approximately 0.5 μM each. The reaction mixture was incubated for 40 cycles in a programmable heat block (Techne Instruments, Ltd., OSI, Paris, France) for 1 min at 93°C, 1 min at the annealing temperature (55°C), and 2 min at 72°C.

Amplified PCR fragments were digested with *Sac*I and *Hin*dIII, whose restriction sites are present in oligonucleotide primers I and II, respectively (underlined sequence). They were ligated into M13mp19 (26) and introduced into *E. coli* JM101 by transformation. DNA sequencing was carried out by the dideoxy chain termination method of Sanger et al. (32), using the T7 sequencing kit (Pharmacia Biotech, Orsay, France).

Construction of the chimeric plasmid and transformation. The multicopy plasmid pJSW102 was derived from pJSW101 by plating on ceftazidime (33). This plasmid carried the *gyrA* gene and conferred resistance to ceftazidime. Construction of the chimeric plasmid containing the different mutations were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and derivation	Relevant phenotype[a]	Reference
E. coli			
KL16	Wild type	Nal^s FQ^s	33
Q1	Clinical isolate	Nal^s FQ^s	27
Q2	Clinical isolate *gyrA*(Gly81Asp)	Nal^s FQ^r	27
Q3	Selected on Nal from Q_2	Nal^r FQ^r	This study
KNK453	*gyrA43*(Ts) *polA thiA uvrA thx*	Nal^s FQ^s	19
KF130	K16 *gyrA* (Ser83Leu)	Nal^r FQ^r	33
CJ236	F′ *dut ung*		21
JM101	F′ *traD36 proAB lac*I^q Z DM15 *thi strA endA hsdR sbcB15 supE*		14
Plasmids			
pJSW101	Derivative of pUC19 with *gyrA* (wild type) from *E. coli* KL16	Amp^r	33
pJSW102	Derivative of pJSW101, conferring resistance to ceftazidime	Caz^r	This study
pJSW103	Derivative of pJSW102 with a 418-bp *Acc*I-*Bam*HI fragment of *gyrA* containing mutation Gly81Asp from *E. coli* Q_2	Caz^r	This study
pJSW104	Derivative of pJSW102 with a 418-bp *Acc*I-*Bam*HI fragment of *gyrA* containing mutation Asp82Gly	Caz^r	This study
pJSW105	Derivative of pJSW102 with a 418-bp *Acc*I-*Bam*HI fragment of *gyrA* containing mutation Ser83Leu from *E. coli* KF130	Caz^r	This study
pJSW106	Derivative of pJSW102 with a 418-bp *Acc*I-*Bam*HI fragment of *gyrA* containing mutation Gly81 Asp Asp82Gly from *E. coli* Q3	Caz^r	33, this study

[a] Nal, nalidixic acid; FQ, fluoroquinolones; Amp, ampicillin; Caz, ceftazidime; s, susceptible; r, resistant.

carried out as described by Soussy et al. (33) with the PCR amplified fragments digested with *Acc*I-*Bam*HI. Transformation was achieved by electroporation, and transformants were selected on BHI agar containing ceftazidime at 4 μg/ml.

Site-directed mutagenesis. Mutagenesis was performed by the uracil template method (21) (Altered Sites kit; Promega, Madison, Wis.). Single-stranded DNA was prepared from the M13mp18 vector containing the 668-bp *Sac*I-*Hin*dIII *gyrA* fragment carrying the QRDR. *E. coli* CJ236 (*dut ung*) carrying the vector M13mp18 with the insertion was used to produce single-stranded DNA containing uracil by the method of McClary et al. (25). In vitro DNA synthesis was performed with uracil single-stranded DNA, and the 29-mer oligonucleotide primer 5′GGTAGACCGCCGAGCCACCATGGGGATGG 3′ containing the desired mutation at position 245 (underlined) to encode the Asp82Gly substitution. The DNA was introduced into *E. coli* JM101, mixed with soft agar, and overlaid onto minimal agar plates. The single-stranded form of the recombinant DNA, extracted from plaques of transfected JM101, were analyzed by DNA sequencing to confirm the presence of the appropriate mutation.

The construction of the chimeric plasmid containing the Asp82Gly mutation was carried out as described by Soussy et al. (33). The novel plasmid, in which the *Acc*I-*Bam*HI *gyrA* fragment was replaced by that present in M13mp18, is derived from pJSW102. The recombinant plasmid was named pJSW104 (Table 1).

Purification of DNA gyrase and supercoiling assays. The purification procedure was carried out as previously described (2) with 2 liters of culture of *E. coli*, with minor modifications. The 37 to 42% ammonium sulfate fraction was dialyzed overnight against 1 liter of buffer (25 mM HEPES-KOH, 200 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% [vol/vol] ethylene glycol, pH 8). The dialysate was applied to a column of novobiocin-sepharose equilibrated with the same buffer. The holoenzyme was eluted with 20 mM ATP–25 mM acetate, pH 4, and the fractions were pooled and concentrated with polyethylene glycol 20000. Protein concentrations were measured according to the procedure of Bradford (5), and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described by Laemmli (22). DNA supercoiling assays were carried out as previously described (2, 11) to detect the gyrase activity. The standard reaction mixture for assaying the supercoiling activity of DNA gyrase contained 35 mM Tris-HCl (pH 7.5), 20 mM KCl, 20 mM $MgCl_2$, 1 mM EDTA, 2 mM spermidine, 1 mM ATP, 0.5 mM DTT, 30 μg of bovine serum albumin (BSA) per ml, 10% ethylene glycol, 100 ng of relaxed pM2 phage DNA from Gibco BRL (Life Technologies, European Division, Eragny, France) and 1 U of gyrase in a 30-μl assay volume (1 U of enzyme was defined as the minimum amount required to supercoil 100 ng of relaxed pM2 DNA in 30 min at 37°C). After 30 minutes at 37°C, 30 μl of stop solution containing 2% SDS, 10 mM EDTA, 0.1% bromophenol blue, 20% glycerol, and 0.4 μg of proteinase K per ml was added. The reaction products were separated on a 0.8% agarose gel, stained with 0.5 μg of ethidium bromide per ml, and photographed under UV light. The minimal dose of quinolones able to inhibit gyrase (MED) was determined by using electrophoretic assay, with various concentrations of quinolones and a constant concentration of gyrase (2).

For the purification of the DNA gyrase with the Asp82Gly mutation, we used *E. coli* KNK453 *gyrA* (Ts) (19) transformed with plasmid pJSW104 harboring the desired mutation. Bacteria were grown at 42°C, and the inhibition test was performed at 42°C to ensure the inactivation of the chromosome-encoded GyrA subunit of the gyrase.

Ethidium bromide fluorescence measurements for the determination of kinetic constants of DNA gyrase. The ethidium fluorescence assay used here has previously been described (1, 23, 28). The assay buffer contained 20 mM potassium phosphate (pH 12), 0.5 mM EDTA, and 0.5 μg of ethidium bromide per ml and was kept at room temperature in a light-proof container. A Hitachi fluorimeter with an excitation filter at 525 nm and an emission filter at 600 nm was used to measure the fluorescence. Before the samples were read, the fluorimeter was zeroed with a blank of assay buffer and then standardized to 50 arbitrary fluorescence units with the addition of 50 μl of pM2 supercoiled DNA (10 μg/ml) to 2 ml of assay buffer. The supercoiling of relaxed pM2 DNA was followed by the concomitant increase in ethidium fluorescence, for which an increase of 40% corresponds to complete supercoiling. After incubation of the standard supercoiling mixture as described above, 15 μl of the reaction mixture was removed and added to 2 ml of assay buffer, and the fluorescence was measured. The remaining 15 μl of reaction mixture was submitted to the standard electrophoresis assay.

To determine the rates of supercoiling of *E. coli* gyrases we followed the time course of gyrase-catalyzed DNA supercoiling monitored by ethidium bromide fluorescence, as described above, using various DNA concentrations. Initial rates were determined from six individual time points taken in the early phase of the reaction (less than 10 min).

RESULTS

In vitro selection of the double mutant *E. coli* Q_3 from *E. coli* Q_2. By plating Q_2, which harbors the Gly81Asp mutation, onto 32 μg of nalidixic acid per ml, we selected a nalidixic acid-resistant mutant, Q_3, at a frequency of ca. 10^{-8} to 10^{-9}. The MICs of quinolones for *E. coli* Q_1, Q_2, Q_3, and KF130 harboring the Ser83Leu mutation, which was used as a reference resistant mutant, are shown in Table 2. The double mutant Q_3 was highly resistant both to nalidixic acid (MIC = 128 μg/ml) and to ciprofloxacin (MIC = 16 μg/ml).

Nucleotide sequence analysis. Nucleotide sequencing of the 668-bp fragment of the *gyrA* gene of Q_3 revealed a second transition (A→G) at nucleotide 245, leading to the replacement of the aspartic acid at position 82 by a glycine in addition to the Gly81Asp mutation already present in Q_2. Asp82Gly has not been described previously and resulted in the substitution of the neutral amino acid Gly for the negatively charged Asp.

Role of the Asp82Gly mutation. In Q_3, the Asp82Gly mutation, associated with the Gly81Asp mutation, engendered resistance to nalidixic acid. To study the phenotype conferred by the sole mutation Asp82Gly, site-directed mutagenesis was used to obtain a fragment of the *gyrA* gene containing the transition A→G at nucleotide 245 and encoding a GyrA with a Gly at position 82. The fragment was then used to construct the chimeric plasmid pJSW104 (Table 1). Table 2 shows the phenotypes obtained at the restrictive temperature (42°C), when KNK453 was transformed with a plasmid encoding the wild-type GyrA subunit and chimeric plasmids encoding the GyrA subunits harboring mutations Gly81Asp, Asp82Gly, or both. When alone on plasmids pJSW103 or pJSW104, either mutation conferred the same phenotype, Nal^S, with a low level of fluoroquinolone resistance, i.e., a four- to eightfold increase in the MIC of ciprofloxacin compared to that for KNK453. When both mutations were present on the plasmid pJSW106, KNK453 became highly resistant to nalidixic acid (MIC = 128

TABLE 2. MICs of quinolones for *E. coli* strains harboring plasmids carrying different *gyrA* alleles

Recipient cells[a]	MIC (μg/ml)[b] with: transforming plasmid											
	None		pJSW102 (WT)		pJSW105 (Leu83)		pJSW103 (Asp81)		pJSW104 (Gly82)		pJSW106 (Asp81Gly82)	
	Nal	Cip	Nal	Cip	Nal	Cip	Nal	Cip	Nal	Cip	Nal	Cip
KNK453 (*gyrA*^{ts})[c]	4	0.06	2	0.015	128	0.125	8	0.25	8	0.5	128	1
KL16 (WT)	2	0.015	2	0.015	64	0.06	2	0.015	2	0.015	2	0.03
KF130 (**Leu**83)	128	0.125	2	0.015	128	0.125	16	0.125	128	0.125	128	0.5
Q_1 (WT)	2	0.015	2	0.015	64	0.06	2	0.015	2	0.015	2	0.03
Q_2 (**Asp**81)[d]	4	8	4	0.5	64	4	4	8	4	8	64	16

[a] Amino acids present at positions 81, 82, and 83 are indicated in parentheses; amino acids differing from the wild type (WT) are in boldface.
[b] When a plasmid was present, MICs were determined on BHI agar plates containing 4 μg of ceftazidime per ml to ensure its stability. Nal, nalidixic acid; Cip, ciprofloxacin.
[c] For KNK453, MICs were determined at 42°C, except for KNK453 without plasmid (30°C), which did not grow at 42°C. *gyrA*^{ts}, thermosensitive gyrase.

μg/ml), while the MIC of ciprofloxacin increased to 1 μg/ml. The difference in the level of resistance observed for ciprofloxacin in KNK453, after introduction of these different plasmids encoding gyrases harboring the simple mutations 81 and 82 and the double mutation 81 and 82, and that observed in the parental strains Q_2 and Q_3 is due to the additional impaired accumulation affecting the fluoroquinolone but not nalidixic acid, present in these later strains (reference 28 and data not shown). As a control, plasmid pJSW105 (Ser83Leu), constructed by using the QRDR from KF130 *gyrA*, was introduced into KNK453, where it produced the same phenotype as that of the parental strain.

The MICs of the quinolones for KNK453 transformed with the different plasmids were the same at 42°C and at the permissive temperature (30°C). A similar observation was made previously (9, 39) and suggests that the thermosensitive *gyrA* gene of KNK453 has a recessive expression at 30°C.

Enzymatic properties of the mutant gyrases. The enzymatic activities of the gyrases isolated from the quinolone-resistant mutants Q_2, Q_3, and KF130 and from KNK453 Asp82Gly (pJSW104) were compared with that of the wild-type gyrase from KL16. The enzymes are ca. 80% pure and contain equal amounts of A and B subunits as revealed by SDS-polyacrylamide gel electrophoresis (data not shown). The specific activities of these holoenzymes were in the range of 0.1×10^4 to 0.25×10^4 U/mg. The MED as determined by using the electrophoresis assay are shown in Table 3. The MED of ciprofloxacin for the double mutant Q_3 was only 2-fold higher than those observed for Q_2 and Asp82Gly, while the MED for nalidixic acid was 30-fold higher, consistent with the results of the complementation test on the role of the double mutation. Interestingly, the MED of ciprofloxacin for Q_2 and Q_3 enzymes were in the range of that of KF130, which confirmed the role of the impaired accumulation as the additional mechanism responsible for the high-level fluoroquinolone resistance observed in the former. We determined the rates of DNA supercoiling from several time points taken in the initial phase of reaction using the fluorescence assay described above. Figure 1 shows the time course of *E. coli* KL16 gyrase-catalyzed supercoiling of five different concentrations of DNA. The same result was obtained with the four other gyrases (not shown).

Complementation tests. Transformation with pJSW102 bearing *gyrA* (wild type) restored the susceptibility to nalidixic acid and fluoroquinolones of all the strains (Table 2). However, strains Q_2 and Q_3 remained somewhat resistant to fluoroquinolones, probably because of the impaired accumulation (28). We were interested in seeing whether the susceptibility to nalidixic acid conferred by the mutations Gly81Asp and Asp82Gly could be also dominant over resistant alleles, when carried on a multicopy plasmid. Therefore, additional merodiploids were constructed by using different hosts and the chimeric plasmids pJSW103 (Gly81Asp), pJSW104 (Asp82Gly), pJSW105 (Ser83Leu), and pJSW106 (Gly81Asp Asp82Gly). Table 2 shows that transformation with pJSW105 (Ser83Leu) conferred resistance to nalidixic acid to all the strains, including the wild-type, KL16. To exclude the possibility of recombination of plasmid-borne *gyrA* (encoding resistance) (Ser83 Leu) with the chromosome, the transformation of KL16 was repeated several times; five clones were isolated each time, giving the same resistance phenotype. Furthermore, when grown on plates devoid of ceftazidime, the transformants tend to lose the plasmid, and colonies susceptible to ampicillin and nalidixic acid may be recovered (data not shown). Transformation with pJSW103 (Gly81Asp) caused no change in the phenotype of the wild-type strain but lowered the resistance to nalidixic acid of Q_3 and KF130 by factors of 4 and 8, respectively. Transformation with pJSW104 (Asp82Gly) did not alter the phenotype of any strain, except KNK453, which appears to be always recessive. Transformation with pJSW106 (Gly81Asp Asp82Gly) did not modify the susceptibility of the wild-type strain but rendered Q_2 resistant to nalidixic acid.

DISCUSSION

In *E. coli*, *gyrA* mutations conferring resistance to quinolones are located within a 130-bp fragment, the QRDR, centered around codon 83, which is the most frequently altered in resistant clinical isolates (6, 16, 17, 35). Starting from an *E. coli* clinical isolate, Q_2, harboring a mutation at position 81 of the DNA gyrase (Gly81Asp) associated with a particular phenotype (Nal^s FQ^r) (6), we selected, on nalidixic acid, a double mutant named Q_3. This mutant was highly resistant to nalidixic

TABLE 3. MICs and MED of quinolones for *E. coli* strains[a]

Strains	MIC (μg/ml)		MED (μg/ml)	
	Nal	Cip	Nal	Cip
KL16	2	0.015	20	0.08
Q1	2	0.015	20	0.08
Q2	4	8	20	20
KNK453 (*gyr*A82Gly)	8	0.5	20	40
Q3	128	16	640	40
KF130	128	0.12	640	20

[a] Nal, nalidixic acid; Cip, ciprofloxacin.

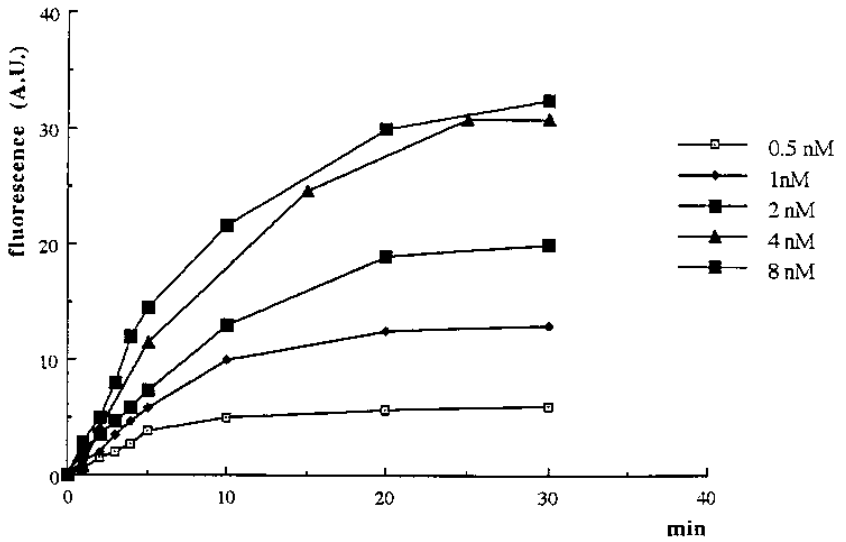

FIG. 1. Monitoring of the time course of gyrase-catalyzed DNA supercoiling activity at a range of DNA concentrations. Samples (15 μl) were taken at different times from the supercoiling reaction (0 to 30 min) and were analyzed by BET-fluorescence. The results shown are for KL16, and the same were obtained with the four other gyrases: gyrase harboring mutations (Asp81Asp82), (Gly81Gly82), (Asp81Gly82), and (Leu83). A.U., arbitrary units.

acid and fluoroquinolones and had a novel mutation, Asp82 Gly, in addition to Gly81Asp of Q_2.

Since strains Q_2 and Q_3 have an impaired accumulation of fluoroquinolones, we used transformation of *E. coli* KNK453, which has a thermosensitive gyrase A, to assess the sole effect of the *gyrA* mutations at the restrictive temperature. Transformation of *E. coli* KNK453 with a chimeric plasmid encoding the double mutant Gly81AspAsp82Gly, conferred high-level resistance to nalidixic acid and moderate resistance to fluoroquinolones and, separately, the mutation Gly81Asp and Asp82 Gly, gave a similar nalidixic acid-susceptible and low-level-fluoroquinolone-resistant phenotype. Therefore, the presence of both mutations in the GyrA subunit are necessary for the resistance to nalidixic acid. This situation, to our knowledge, has not been previously encountered since, generally, double mutations yielding high-level resistance to fluoroquinolones have resulted from single mutations which alone conferred resistance to nalidixic acid (16, 35).

The results of MIC testing were confirmed by the isolation of the corresponding gyrases and their assay for inhibition of DNA supercoiling by nalidixic acid and ciprofloxacin. The mutations at position 81 or 82 did not alter the sensitivity of the enzyme to inhibition by nalidixic acid but conferred a low level of resistance to ciprofloxacin, whereas the gyrase containing both mutations was highly resistant to all the quinolones. It is noteworthy that the rates of supercoiling for the different enzymes at a range of DNA concentration were comparable. This indicated that these gyrases interact normally with DNA and that the single or double mutations described in this report did not significantly alter the supercoiling activity of the enzymes. Therefore, the amino acids within the QRDR could play a role in the association of gyrase with quinolones but not in the activity of the holoenzyme itself.

We also examined the issue of dominance among different alleles of *gyrA*. As previously described (15), when introduced into any resistant mutant, the wild-type *gyrA* gene reversed the resistance phenotype; thus, nalidixic acid-susceptible *gyrA* alleles are dominant over nalidixic acid-resistant ones. This dominance of the susceptible allele could result from preferential expression of the wild-type gene product, because the mutant protein either is less efficient in its catalytic properties, is translated poorly, or is degraded more readily by proteases (19). It may also relate to the number of gene copies within the cell; if this were the case, the plasmid-encoded gyrase, even if it is a mutant, would be dominant over the chromosome-encoded one. Therefore, we introduced the multicopy plasmid pJSW105 encoding the Ser83Leu mutant gyrase into the various hosts used in this study. The results (Table 2) are in agreement with the hypothesis of gene dosage, since the resistance to nalidixic acid conferred by the Ser83Leu mutant *gyrA* was always dominant. Such a result has been reported by Gomez-Gomez et al. (12), who introduced an unidentified nalidixic acid resistance-encoding *gyrA* gene into *E. coli* KL16 cell. Cullen et al. (9) pointed out that KNK453 (Ts), transformed with a plasmid coding for a highly resistant gyrase (Ser83Trp), was highly resistant, even at the permissive temperature. The same phenomenon was observed by Yoshida et al. (38), although no explanation on a molecular basis of this dominance was given. In our study we observed that the introduction of any plasmid harboring a *gyrA* gene into KNK453 imposed its phenotype, even at the permissive temperature (30°C). This is also in good agreement with the hypothesis of dominance of the multicopy plasmid-encoded gyrase over the unique chromosome-encoded one.

However, the situation became more complex when other hosts were transformed with plasmids encoding the single *gyrA* mutant Gly81Asp and Asp82Gly or the double *gyrA* mutant Gly81Asp Asp82Gly. Both single mutations conferred a nalidixic acid-susceptible phenotype and a low-level resistance to ciprofloxacin (four- to eightfold increase) in KNK453. When introduced into the hosts used in this study, the plasmid encoding the susceptible phenotype associated with Asp82Gly had no effect, suggesting that in this case the chromosome-encoded gyrases were dominant and that Asp82Gly was recessive although encoded by a multicopy plasmid. In contrast, the presence of the plasmid pJSW103 (Gly81Asp) was able to lower the resistance to nalidixic acid of Q3 and of KF130, although it did not restore a full susceptibility, suggesting that the nalidixic acid-susceptible phenotype associated with Gly81Asp was only partially dominant. Finally, the nalidixic

acid and fluoroquinolone resistance conferred by the double mutation Gly81Asp Asp82Gly was obviously recessive with respect to the chromosomally encoded wild-type phenotype of KL16. However, it was dominant over the chromosomal mutation Gly81Asp in Q_2, where it increased the resistance to nalidixic acid.

The results of transformation using *gyrA*$^+$ and *gyrA* (encoding resistance) (Ser83Leu) are in good agreement with the hypothesis of dominance of the subunit in excess dosage and not of the *gyrA*$^+$ over the *gyrA* one. However, the effect of transformations by plasmids harboring mutations at position 81, 82, or both are compatible with neither hypothesis. Since the rates of supercoiling of all the gyrases of this study were similar, the dominance cannot be explained by enzyme efficacy. Thus, it is very likely that both the nature and position of the mutation in the QRDR, as well as its location on the chromosome or on a multicopy plasmid, are critical for the expression of dominance. One possible explanation of the observed pattern of dominance is that critical amino acids in the QRDR play a role in the association of gyrase subunits. Recent results (3) on the structure and mechanism of DNA topoisomerase II based on the crystal structure of the yeast enzyme indicated that in the dimeric enzyme, contacts between the A and B domains of each promoter occur primarily in the region corresponding to the QRDR of the procaryotic DNA gyrase. This is consistent with our hypothesis of the importance of the QRDR in the association of the A and B subunits. We hypothesize that after transformation of a strain encoding a chromosomal gyrase, $A^c{}_2B_2$, by a plasmid coding for another type of gyrase A subunit, A^p, there may be within the cell several types of gyrases, due to the association of the different subunits A and B: $A^c{}_2B_2$, $A^p{}_2B_2$, and $A^cA^pB_2$. If the plasmidic GyrA subunit, A^p, is overexpressed, then there will be essentially no homodimers of the chromosomic GyrA subunit, $A^c{}_2$, only a small number of heterodimers A^cA^p, and the phenotype of the overexpressed A^p subunit will be dominant. If both GyrA subunits are expressed at similar levels, the result will depend on the stabilities of the different possible A_2B_2 gyrases. At this time, the formation, the enzymatic behavior, and the inhibition by a quinolone of the heterodimer $A^cA^pB_2$ remain hypothetical. In this context, Kreuzer and Cozzarelli (19) suggested that when both sensitive and resistant promoters exist in one tetramer, inactivation of any one protomer by an inhibitor may block the activity of the multimer.

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REFERENCES

1. **Andrea, J. A., K. Adachi, and R. Morgan.** 1991. Fluorometric assays for DNA topoisomerases and topoisomerase-targeted drugs: quantitation of catalytic activity and DNA cleavage. Mol. Pharmacol. **40:**495–501.
2. **Bazile, S., N. J. Moreau, D. Bouzard, and M. Essiz.** 1992. Relationships among antibacterial activity, inhibition of DNA gyrase, and intracellular accumulation of 11 fluoroquinolones. Antimicrob. Agents Chemother. **36:** 2622–2627.
3. **Berger, J. M., S. J. Gamblin, S. C. Harrison, and J. C. Wang.** 1996. Structure and mechanism of DNA topoisomerase II. Nature **379:**225–232.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **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.
6. **Cambau, E., F. Bordon, E. Collatz, and L. Gutmann.** 1993. Novel *gyrA* point mutation in a strain of *Escherichia coli* resistant to fluoroquinolones but not to nalidixic acid. Antimicrob. Agents Chemother. **37:**1247–1252.
7. **Chu, D. T. W., and P. B. Fernandes.** 1989. Structure-activity relationships of the fluoroquinolones. Antimicrob. Agents Chemother. **33:**131–135.
8. **Crumplin, G. C.** 1990. Mechanisms of resistance to the 4-quinolone antibacterial agents. J. Antimicrob. Chemother. **26:**131–144.
9. **Cullen, M. E., A. W. Wyke, R. Kuroda, and L. M. Fischer.** 1989. Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers clinical resistance to 4-quinolones. Antimicrob. Agents Chemother. **33:**886–894.
10. **Denis, A., and N. J. Moreau.** 1993. Mechanisms of quinolone resistance in clinical isolates: accumulation of sparfloxacin and of quinolones of various hydrophobicity, and analysis of membrane composition. J. Antimicrob. Chemother. **32:**379–392.
11. **Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash.** 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73:**3872–3876.
12. **Gomez-Gomez, J. M., F. Baquero, M. R. Baquero, J. C. Perez-Diaz, and J. Blazquez.** 1995. Quinolone resistance conferred by an engineered multicopy plasmid containing a dominant *gyrA* mutation, abstr. C60, p. 50. *In* Program and abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
13. **Hallett, P., and A. Maxwell.** 1991. Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of the mutant proteins. Antimicrob. Agents Chemother. **35:**335–340.
14. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
15. **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.
16. **Heisig, P., H. Schedletzky, and H. Falkenstein-Paul.** 1993. Mutations in the *gyrA* gene of a highly fluoroquinolone-resistant clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **37:**696–701.
17. **Heisig, P., and R. Tschorny.** 1994. Characterization of fluoroquinolone-resistant mutants of *Escherichia coli* selected in vitro. Antimicrob. Agents Chemother. **38:**1284–1291.
18. **Horowitz, D. S., and J. C. Wang.** 1987. Mapping the active site tyrosine of *Escherichia coli* DNA gyrase. J. Biol. Chem. **262:**5339–5344.
19. **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:**524–535.
20. **Kumagai, Y., J. I. Kato, K. Hoshino, T. Akasaka, K. Sato, and H. Ikeda.** 1996. Quinolone-resistant mutants of *Escherichia coli* DNA topoisomerase IV *parC* gene. Antimicrob. Agents Chemother. **40:**710–714.
21. **Kunkel, T. A., J. D. Roberts, and R. A. Zakow.** 1987. Rapid and efficient site-specific mutagenesis without phenotype selection. Methods Enzymol. **154:**367–382.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
23. **Liu, L. F., and J. C. Wang.** 1978. *Micrococcus luteus* DNA gyrase: active components and a model for its supercoiling of DNA. Proc. Natl. Acad. Sci. USA **75:**2098–2102.
24. **Maxwell, A., and M. Gellert.** 1984. The DNA dependence of the ATPase activity of DNA gyrase. J. Biol. Chem. **259:**14472–14480.
25. **McClary, J. A., F. Witney, and J. Geisselsoder.** 1989. Efficient site-directed in vitro mutagenesis. BioTechniques **7:**282–287.
26. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
27. **Moniot-Ville, N., J. Guibert, N. J. Moreau, J. F. Acar, E. Collatz, and L. Gutmann.** 1991. Mechanisms of quinolone resistance in a clinical isolate of *Escherichia coli* highly resistant to fluoroquinolones but susceptible to nalidixic acid. Antimicrob. Agents Chemother. **35:**519–523.
28. **Morgan, A. R., D. H. Evans, J. S. Lee, and D. E. Pulleyblank.** 1979. Review: ethidium fluorescence assay. Part II. Enzymatic studies and DNA-protein interactions. Nucleic Acids Res. **7:**571–594.
29. **Oram, M., and L. M. Fisher.** 1991. 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. Antimicrob. Agents Chemother. **35:**387–389.
30. **Robillard, N. J.** 1990. Broad-host-range gyrase A gene probe. Antimicrob. Agents Chemother. **34:**1889–1894.
31. **Rosen, T.** 1990. The fluoroquinolone antibacterial agents. Prog. Med. chem. **27:**235–295.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
33. **Soussy, J. C., J. S. Wolfson, E. Y. Ng, and D. C. Hooper.** 1993. Limitations of plasmid complementation test for determination of quinolone resistance due to changes in the gyrase A protein and identification of conditional quinolone resistance locus. Antimicrob. Agents Chemother. **37:**2588–2592.
34. **Truong, Q. C., L. Gutmann, D. Shlaes, and N. J. Moreau.** 1995. New

mutation in DNA gyrase of *E. coli* conferring resistance to quinolones, abstr. C61, p. 50. *In* Program and abstracts of the Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.

35. **Vila, J., J. Ruiz, F. Marco, A. Barcelo, P. Goni, E. Giralt, and T. Jimenez De Anta.** 1994. Association between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. Antimicrob. Agents Chemother. **38:**2477–2479.
36. **Willmott, C. J. R., and A. Maxwell.** 1993. A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. Antimicrob. Agents Chemother. **37:**126–127.
37. **Yamagishi, J. I., 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.
38. **Yoshida, H., T. Kojima, J. I. Yamagishi, and S. Nakamura.** 1988. Quinolone-resistant mutations of the *gyrA* gene of *Escherichia coli*. Mol. Gen. Genet. **211:**1–7.
39. **Yoshida, H., M. Nakamura, M. Bogaki, H. Ito, T. Kojima, H. Hattori, and S. Nakamura.** 1993. Mechanism of action of quinolones against *Escherichia coli* DNA gyrase. Antimicrob. Agents Chemother. **37:**839–845.