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Salmonella typhimurium gyrA Mutations Associated with Fluoroquinolone Resistance

FERNANDO REYNA, MARIO HUESCA,† VÍCTOR GONZÁLEZ,‡ AND L. YOLANDA FUCHS*

Centro de Investigaciones sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Cuernavaca, Morelos 62508, Mexico

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Spontaneous quinolone-resistant mutants obtained from *Salmonella typhimurium* Su694 were screened for mutations by direct DNA sequencing of an amplified PCR *gyrA* fragment. Substitutions Ser-83→Phe (Ser83Phe), Ser83Tyr, Asp87Tyr, and Asp87Asn and double mutation Ala67Pro-Gly81Ser, which resulted in decreased sensitivities to ciprofloxacin, enoxacin, pefloxacin, norfloxacin, ofloxacin, and nalidixic acid, were found. The levels of resistance to quinolones for each mutant were determined.

Fluoroquinolones are potent antibacterial agents, derived from nalidixic acid, that inhibit DNA gyrase (11, 12). Active DNA gyrase is a tetramer composed of two A and two B subunits, with molecular masses of 97 and 90 kDa for each monomer, respectively (17, 26), encoded by the *gyrA* and *gyrB* genes (10, 12). This enzyme maintains bacterial DNA in a negatively supercoiled state by utilizing magnesium and ATP (7). *gyrA* sequences in *Escherichia coli* (35), *Klebsiella pneumoniae* (6), *Bacillus subtilis* (27), *Mycoplasma pneumoniae* (4), *Staphylococcus aureus* (1, 19, 24), *Pseudomonas aeruginosa* (23), *Campylobacter jejuni* (38), *Mycobacterium tuberculosis* (36), *Haloferax* spp. (18), and *Enterococcus faecalis* (22) have been determined.

DNA gyrase activity can be selectively inhibited by quinolones (8, 25). Apparently, quinolones bind to the gyrase-DNA complex at the site of cleaved DNA, where they interact with the single-stranded DNA region (20, 25). It has been proposed that magnesium ions are needed for quinolone-DNA binding and possibly for formation of the quinolone-gyrase-cleavable DNA complex (30). Single point mutations in *gyrA* that confer resistance to quinolone drugs have been described for a variety of microorganisms (14, 21–23, 31, 32, 36, 38). These mutations occur in a conserved region of the N-terminal domain of the A subunit close to the catalytic Tyr-122 site, termed the quinolone resistance-determining region (QRDR).

In *E. coli*, replacement of Ser-83 by Leu or Trp confers high-level resistance to quinolones. Mutations in other residues also result in resistance phenotypes (29, 39).

Fluoroquinolone resistance is rarely found among *Salmonella* species. A clinical isolate of *Salmonella typhimurium* in which resistance to ciprofloxacin was associated with alterations in both gyrase subunits has recently been described (15). The exact locations and genetic changes associated with these mutations have not been defined.

In this study, we analyzed the DNA sequences of *gyrA* fragments from spontaneous resistant mutants of *S. typhimurium* Su694 and their corresponding fluoroquinolone susceptibilities. Strain Su694 is a derivative of *S. typhimurium* LT2 (described elsewhere [2]). Spontaneous mutants were obtained by plating an overnight culture of Su694 on nalidixic acid- or pefloxacin-containing agar. The quinolone concentrations used were 10 and 20 times the MIC for Su694 (9). Several individual colonies were collected from these plates to purify DNA and amplify an 800-bp *gyrA* fragment containing the QRDR. Primers for PCR amplification were designed on the basis of the known *E. coli gyrA* sequence (35) and correspond to nucleotides 93 to 110 and 874 to 890 for forward and reverse reactions, respectively.

PCR amplifications were performed according to the manufacturer's instructions, with 100 ng of DNA template and final concentrations of 2 mM $MgCl_2$, 200 μM (each) deoxynucleotide triphosphate, 10 pmol of each primer, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Reactions were carried out with a Techne PHC-2 cycler (Bio-Synthesis, Inc., Denton, Tex.) and consisted of 5 cycles at 94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min; 25 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min; and a final cycle at 72°C for 15 min. Five microliters of reaction mixture was analyzed on 2% agarose gels in Tris-borate-EDTA buffer, stained with ethidium bromide, and visualized on a UV transilluminator. PCR products were purified by using Wizard DNA cleanup minicolumns (Promega Corp., Madison, Wis.) as directed by the manufacturer.

PCR products were directly used as templates for sequencing by using CircumVent (New England Biolabs, Inc., Beverly, Mass.) according to the methodology of Sanger et al. (33). Reactions were performed with 1.2 pmol of forward primer, 200 ng of purified PCR product, 20 μCi of α-^{35}S-dATP, and 2 U of Vent DNA polymerase by following the manufacturer's instructions. Sequencing reactions were fractionated on 7% acrylamide–8 M urea gels and analyzed by autoradiography.

Figure 1 illustrates the 207-nucleotide QRDR sequences of the *gyrA* genes of wild-type Su694 (GenBank accession number U21957) and wild-type *E. coli*. The inferred amino acid sequences of the analyzed region in both species are identical. The susceptibility patterns of wild-type Su694 and derived mutants to different quinolones are shown in Table 1. MIC determinations were performed on Mueller-Hinton agar according to the standards of the National Committee for Clinical Laboratory Standards (28).

In this study, mutations were found in the QRDRs of all of the Nal^r and Pef^r strains analyzed. Colonies that carried the

* Corresponding author. Mailing address: CISEI, INSP, Av. Universidad 655, Cuernavaca, Morelos 62508, México. Phone: (52-73) 11-0111, ext. 2434. Fax: (52-73) 17-5485. Electronic mail address: yolanda@pbr322.ceingebi.unam.mx.

† Present address: Instituto de Biotecnología, Universidad Nacional Autónoma de México Campus Morelos, Cuernavaca, Morelos 62270, Mexico.

‡ Present address: Department of Microbiology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada.

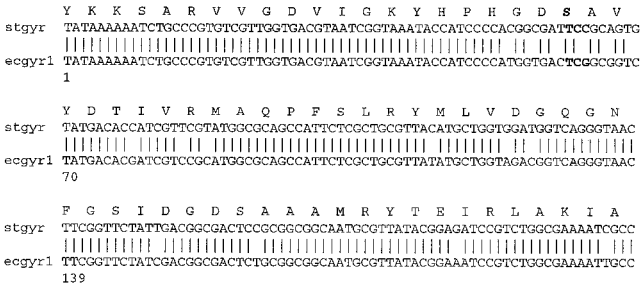

FIG. 1. Nucleotide sequence comparison of the QRDR of *S. typhimurium* Su694 *gyrA* (stgyr) with nucleotides 370 to 577 of *E. coli gyrA* (ecgyr1). The deduced amino acid sequences of this region in both species are identical. Ser-83 is shown in boldface. Vertical lines indicate identical nucleotides.

same mutation and showed the same MICs but had been obtained under different selection conditions (nalidixic acid or pefloxacin at 10 or 20 times the MIC for Su694) were considered to be one strain. Since they were derived from a single bacterial culture, they may represent progeny of an early mutational event.

Strain Su701, with mutation Ser-83→Phe (Ser83Phe), showed the highest resistance levels (Table 1); it had been obtained with pefloxacin at both 10 and 20 times the MIC and with nalidixic acid at 20 times the MIC. This mutation is not found in *E. coli*; spontaneous mutations associated with fluoroquinolone resistance usually correlate with single-base substitutions. Since Ser-83 in *E. coli* is encoded by TCG, it would require two point mutations to be changed into Phe (TTC or TTT). Note that the second base of the codon is the one that frequently changes to yield high-level resistance, giving rise to bulky and hydrophobic amino acid residues. This has been observed in vitro as well as for clinical isolates of other genera. For example, in *Shigella dysenteriae* and *S. aureus*, the change from Ser-83 (TCG; Ser-84 [TCA] in *S. aureus*) to Leu (TTN) confers high-level resistance (13, 21, 31, 34). Replacement of the equivalent residue by isoleucine in *P. aeruginosa* (23), *Enterococcus faecalis* (22), and *Serratia marcescens* (unpublished observations) (5) is also associated with quinolone resistance. In *Mycobacterium smegmatis* (32) and *Mycobacterium avium* (3), replacement by valine produces a similar effect.

Strain Su702, which contains Ser83Tyr and was obtained with nalidixic acid at 10 times the MIC, shows the same ciprofloxacin resistance that Su701 does, but it is more susceptible to the other fluoroquinolones tested (Table 1). Substitution Ser83Tyr has been introduced into *E. coli* by site-directed mutagenesis (25). However, there is no information concerning the level of resistance associated with this replacement.

Substitution Asp87Asn, also obtained with nalidixic acid at 10 times the MIC, has been reported for *E. coli* (39), *M. tuberculosis* (36), *P. aeruginosa* (23), and *C. jejuni* (38). The level of resistance to ciprofloxacin associated with this mutation in Su703 (Table 1) is comparable to that obtained in *E. coli* (39).

Replacement Asp87Tyr, as observed for Su704 (Table 1), was selected with pefloxacin and nalidixic acid at 10 and 20 times the MIC for Su694, respectively. This mutation has been found in *M. tuberculosis* (36) and *P. aeruginosa* (23).

Differences in the levels of resistance among mutants, probably due to particular amino acid changes, may correlate well with single point mutations, as observed for *E. coli* (39). Mutations in strains Su701 through 705 yielded at least a 64-fold increase in the MIC of nalidixic acid and 8- to 64-fold increases in the MICs of fluoroquinolones (Table 1). The double mutation, Ala67Pro-Gly81Ser, in strain Su705 (obtained with pefloxacin at 20 times the MIC) was an unexpected result, since the selection frequency for single-step mutants of Su694 with pefloxacin at 10 times the MIC is around 10^{-9} to 10^{-10}. In *E. coli* and *S. aureus*, double and triple combinatorial mutations involving residues 83 and 87 of GyrA are associated with much higher resistance levels than those of single mutations (13, 16, 37). However, double-mutant Su705 showed the same susceptibility pattern as that of Su702 (Table 1). It is possible that Ala67Pro alone is responsible for this resistant phenotype, since Gly81Ser is quinolone susceptible. Strain Su706, selected with nalidixic acid at 10 times the MIC, may have originated from an unstable Ala67Pro-Gly81Ser double mutant in which Pro-67 reverted to Ala. The possibility that Gly81Ser was mistakenly introduced by PCR is less likely, since it appeared in three independent colonies. Single mutations in both residues, Ala-67 and Gly-81, have been associated with low-level quinolone resistance in other genera. Replacement Gly81 Cys has been observed in *E. coli* (39) and *M. tuberculosis* (36). In *E. coli*, it confers an eightfold increase in the ciprofloxacin MIC. Substitution Ala67Ser has also been detected in *E. coli*, conferring only a fourfold increase in ciprofloxacin resistance (39).

Gly-81 is conserved very well among bacteria, not only in GyrA but also in topoisomerase IV. Replacement of this residue by serine may be too conservative and weak to provide quinolone resistance and may require a synergistic mutation like Ala67Pro for proper function of the enzyme. The individual contribution of Ala67Pro in regard to quinolone resistance in *S. typhimurium* remains to be determined.

By analogy with known resistance mutations in other species, our results suggest an association between the obtained mutations and this resistance phenotype. Complementation studies with a quinolone-susceptible *E. coli gyrA* gene are under way to verify this hypothesis.

TABLE 1. Antimicrobial susceptibilities of *S. typhimurium* mutants

Strain	Mutation(s)	MIC (μg/ml)[a]							
		NAL	PEF	ENO	CIP	OFL	NOR	TET	CM
Su694	Wild type	8	0.125	0.0625	0.0078	0.0625	0.0625	2	2
Su701	Ser83Phe (TCC→TTC)	>512	4	4	0.5	2	2	4	4
Su702	Ser83Tyr (TCC→TAC)	>512	2	1	0.5	0.5	1	2	2
Su703	Asp87Asn (GAC→AAC)	>512	4	4	0.25	0.5	0.125	4	2
Su704	Asp87Tyr (GAC→TAC)	>512	4	4	0.5	1	1	4	4
Su705	Ala67Pro-Gly81Ser (GCC→CCC and GGC→AGC)	>512	2	1	0.5	0.5	2	2	2
Su706	Gly81Ser (GGC→AGC)	8	0.125	0.0625	0.0078	0.0625	0.0625	2	2

[a] NAL, nalidixic acid; PEF, pefloxacin; ENO, enoxacin; CIP, ciprofloxacin; OFL, ofloxacin; NOR, norfloxacin; TET, tetracycline; CM, chloramphenicol.

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