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Short Communication

Influence of Lineage-Specific Amino Acid Dimorphisms in GyrA on *Mycobacterium tuberculosis* Resistance to Fluoroquinolones

Hyun Kim[1], Chie Nakajima[1], Youn Uck Kim[2], Kazumasa Yokoyama[1], and Yasuhiko Suzuki[1,3]*

[1]*Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020;*
[3]*JST/JICA-SATREPS, Tokyo 120-8666, Japan; and*
[2]*Department of Biomedical Sciences, Sun Moon University, A-San 336-708, Republic of Korea*

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SUMMARY: We conducted in vitro DNA supercoiling assays, utilizing recombinant DNA gyrases, to elucidate the influence of the lineage-specific serine or threonine residue at position 95 of GyrA on fluoroquinolone resistance in *Mycobacterium tuberculosis*. There was little effect of the GyrA-Ala74Ser amino acid substitution on activity of the GyrA-Ser95 gyrase, while activity of the GyrA-Asp94Gly-Ser95 gyrase was reduced. These findings were in striking contrast to previous reports analyzing GyrA with Thr95 and suggest an important impact of the amino acid in the development of fluoroquinolone resistance.

The emergence of multidrug-resistant tuberculosis (MDR-TB) is a significant public health problem that poses a serious threat to global TB control (1). As a result, the need for novel classes of anti-TB drugs has increased, with fluoroquinolones (FQs) becoming the drug of choice for use in MDR-TB treatment (2–5).

FQs are a series of synthetic antimicrobial agents that inhibit bacterial DNA gyrase and topoisomerase IV (6–8). Although most bacteria are known to have both enzymes, *Mycobacterium tuberculosis* lacks topoisomerase IV (4–7), and thus, the sole target of FQs in *M. tuberculosis* is DNA gyrase.

The mechanism underlying development of FQ resistance in *M. tuberculosis* appears to involve amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of the A or B subunit of DNA gyrase. These substitutions, which occur mainly at positions 88, 90, 91, and 94 in GyrA, and at positions 500, 521, and 540 in GyrB, have been shown to imbue *M. tuberculosis* with FQ resistance (9–14). In contrast, the polymorphism at position 95 in GyrA, which encodes a serine or threonine, has been shown to have no influence on FQ-resistance. Hence, these residues are considered to be lineage-specific amino acids. Recently, double mutations at codons 74 and 94 in GyrA QRDRs, causing amino acid substitutions from alanine to serine (Ala74Ser) and Asp94Gly, respectively, were reported in clinical isolates with high-level FQ-resistance in China (14,15). The majority of these isolates belong to the Beijing lineage, carrying the lineage-specific amino acid, threonine, at position 95 in GyrA. In addition, evidence that Ala74Ser enables development of FQ resistance was demonstrated using recombinant DNA gyrase constructed from genomic DNA of a *M. tuberculosis* clinical isolate, also bearing the GyrA-Thr95 polymorphism (17). Interestingly, no FQ-resistant clinical isolates carrying the Ala74Ser mutation have been reported in lineages also carrying GyrA-Ser95.

Here, in order to elucidate the influence of lineage-specific amino acid dimorphisms in GyrA on FQ resistance, we conducted in vitro DNA supercoiling inhibition assays utilizing recombinant DNA gyrases bearing a GyrA-Ser95 residue and its derivatives.

We constructed recombinant DNA gyrase expression plasmids using genomic DNA from *M. tuberculosis* H37Rv expressing Ser95 in GyrA, as described previously (12). Ala74Ser, Asp94Gly, and Ala74Ser-Asp94Gly mutations were introduced into the Ser95 *gyrA* DNA by PCR using pairs of complementary primers containing the mutations of interest (Table 1). Plasmid vectors, pET-20b (+) and pET-19b (Merck KGaA, Darmstadt, Germany) were used to construct 6 × His-tagged *M. tuberculosis* GyrA and GyrB expression plasmids. Expression of GyrA and GyrB in *Escherichia coli* Rosetta-gami 2 and BL21 (DE3) pLysS (Merck KGaA) was induced by treatment with 1 mM isopropyl β-D-1-thio-galactopyranoside (IPTG) at 14°C for 20 h, or at 23°C for 5 h.

Recombinant DNA gyrase subunits in sonicated *E. coli* lysates were trapped on nickel-nitrilotriacetic acid agarose resin (Invitrogen Corp., Carlsbad, Calif., USA) and were eluted with elution buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, and 250 mM imidazole). Eluates were dialyzed twice overnight at 4°C against 1 L gyrase dilution buffer (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 2 mM DTT, 1 mM EDTA), an equal volume of glycerol was added, and samples were used for subsequent assays. The inhibitory effects of FQs on DNA gyrases were assessed by carrying out supercoiling inhibition assays in the presence or absence of increasing concentrations of three different FQs purchased from LKT Laboratories, Inc. (St. Paul, Minn., USA): gatifloxacin (GAT), levofloxacin (LVX), and ciprofloxacin (CIP).

*Corresponding author: Mailing address: Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Kita 20-Nishi 10, Kita-ku, Sapporo 001-0020, Japan. Tel: +81-11-706-9503, Fax: +81-11-706-7310, E-mail: suzuki@czc.hokudai.ac.jp

Table 1. Nucleotide sequence of primers used for introducing amino acid substitutions

Primer name	Sequence (nucleotide position)	Amino acid substitutions to be introduced
ON-001	5′-CGCC AAG TCG TCC CGG TCG GTTG-3′ (210–232)	Ala74Ser *gyrA*
ON-002	5′-CAAC CGA CCG GGA CGA CTT GGCG-3′ (210–232)	Ala74Ser *gyrA*
ON-015	5′-GTCG ATC TAC GGC AGC CTG GTGC-3′ (270–292)	Asp94Gly *gyrA*
ON-016	5′-GCAC CAG GCT GCC GTA GAT CGAC-3′ (270–292)	Asp94Gly *gyrA*
ON-031	5′-GCAACTACCACCCGCACGGC-3′ (245–264)	Ala74Ser-Asp94Gly *gyrA*
ON-032	5′-GCCGTGCGGGTGGTAGTTGC-3′ (245–264)	Ala74Ser-Asp94Gly *gyrA*

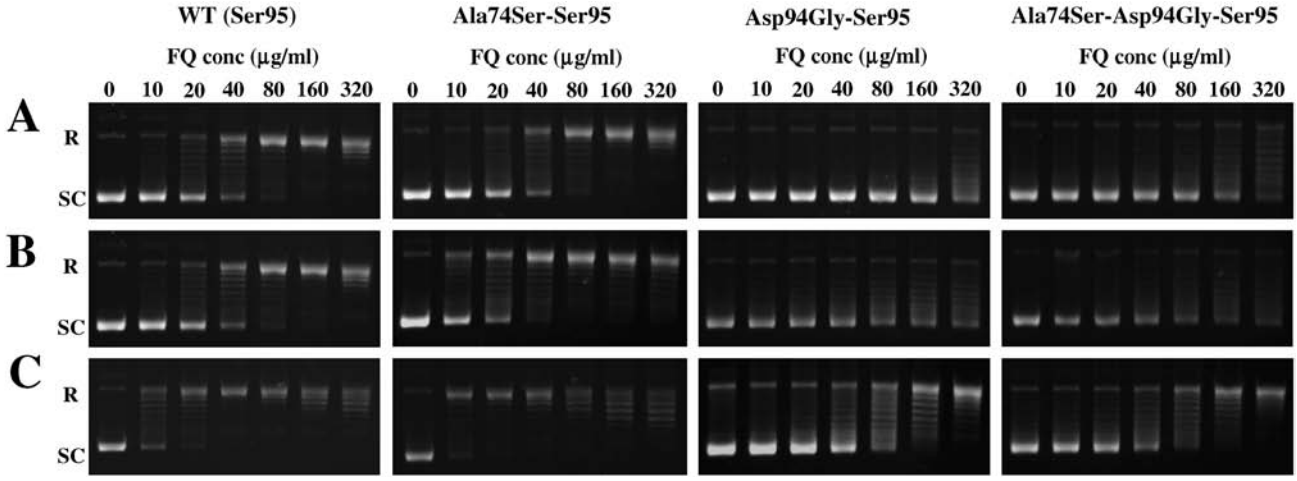

Fig. 1. Fluoroquinolones inhibited DNA supercoiling in assay. Relaxed pBR322 DNA (0.3 μg) was incubated with 3 μM of wild type (WT) or mutant GyrA and 3 μM of GyrB in the presence or absence of the indicated amounts (in μg/ml) of quinolones. A, levofloxacin; B, ciprofloxacin; and C, gatifloxacin at 37°C for 60 min. After stopping the reaction by adding equal volume of chloroform: isoamyl alcohol (24:1), DNA products in aqueous phase were analyzed by electrophoresis in a 1% agarose gel. R and SC indicate relaxed and supercoiled DNA, respectively.

Table 2. IC_{50} of FQs against wild type and mutant DNA gyrases

Quinolone	IC_{50}[1] (μg/ml)						Reference
	WT H37Rv (Ser95)	Ser95Thr	Ala74Ser-Ser95	Ala74Ser-Ser95Thr	Asp94Gly-Ser95	Ala74Ser-Asp94Gly-Ser95	
LVX	34		34		310	171	This work
CIP	18		17		196	107	
GAT	9		7		76	48	
OFX	1.92	3.30		15.56			Lau et al. (17)
MXF	0.98	1.62		14.37			

[1]: IC_{50}: half maximal inhibitory concentrations.
WT, wild type; LVX, levofloxacin; CIP, ciprofloxacin; GAT, gatifloxacin; OFX, ofloxacin; MXF, moxifloxacin.

Drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50} values) were calculated using a formula developed by plotting amounts of supercoiled DNA against FQ concentration, as described previously (6,9,12,16).

Each of the FQ dose-dependently inhibited DNA supercoiling activity with IC_{50} values ranging from 7 to 310 μg/ml (Fig. 1 and Table 2). Wild type (GyrA-Ser95) and GyrA-Ala74Ser-Ser95 demonstrated similar levels of in vitro FQ susceptibility, implying that there is little influence of the GyrA-Ala74Ser amino acid substitution on inducing resistance against FQs. This is in striking contrast to data reported by Lau et al. (17). They noted 8.1- and 14.7-fold increases in resistance to ofloxacin (OFX) and moxifloxacin (MXF), respectively, for DNA gyrase with a GyrA-Ala74Ser substitution compared to that without this substitution in GyrA-Thr95 (Table 2).

The discrepancy between our present findings and those of Lau et al. suggests that Ala74Ser substitution affects FQ resistance only when there is a threonine at position 95 of GyrA. The amino acid at position 74 has been suggested to exist in the α3-helix domain, while that at position 95 is in the α4-helix domain (17–19). In addition, the Ala74Ser amino acid substitution has been hypothesized to perturb the GyrA-GyrA dimer interface by interrupting hydrophobic interactions between the two α3-helix domains, thereby contributing to FQ resistance (17). Our data suggest a possible role for the threonine methyl group at position 95 in this phenomenon by causing altered interactions between α4- and α3-helices, or other nearby structures, followed by induction of conformational changes in the quinolone-bind-

ing pocket (QBP).

Furthermore, we found that the IC_{50} values of LVX, CIP, and GAT against DNA gyrase with GyrA-Asp94Gly-Ser95 were 2-fold greater than those against DNA gyrase with GyrA-Ala74Ser-Asp94Gly-Ser95 (Table 2). In contrast, two previous studies have reported that Ala74Ser enhances FQ resistance. Shi et al. analyzed OFX-resistant clinical isolates and showed that Ala74Ser-Asp94Gly substitutions in GyrA were associated with a relatively high MIC for OFX, ranging from 2 to 32 μg/ml, whereas GyrA-Asp94Gly had a relatively low MIC, ranging from 1 to 4 μg/ml (15). Similarly, Sun et al. reported that the MICs of OFX against clinical isolates ranged from 4 to 20 μg/ml for those with GyrA-Ala74Ser-Asp94Gly, and from 1 to 10 μg/ml for those with GyrA-Asp94Gly (14). Since Aubry et al. demonstrated a significant correlation ($R^2 = 0.9$) between IC_{50} values for DNA gyrase and the MICs of FQs (6), the striking contrast between the findings of the two previous publications and our current data regarding the influence of GyrA-Ala74Ser and GyrA-Asp94Gly in GyrA-Ser95 suggests that the threonine at position 95 may enhance resistance by altering interactions between the α4- and α3-helices, or other structures. Furthermore, the high level of FQ resistance found in the Beijing genotype reported from Vietnam (20), which also carries a threonine at position 95, suggests an influence of lineage-specific amino acid residues on the acquisition of enhanced resistance.

In conclusion, the lineage-specific amino acid residue at position 95 in GyrA enables the acquisition of high-level resistance in *M. tuberculosis* against FQs, likely by inducing conformational changes in the QBP. Further studies of *M. tuberculosis* resistance, with various combinations of amino acid substitutions in DNA gyrases, are needed to confirm these observations.

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Conflict of interest None to declare.

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